

Chemical Modification and Inhibition
of Liver Alcohol Dehydrogenase

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Degree of Doctor of Philosophy

University of Edinburgh

1972



Contents

Preface	i
Summary	ii
Abbreviations	v
<u>Chapter 1</u>	1
Introduction								
<u>Chapter 2</u>	14
Materials and Methods								
<u>Chapter 3</u>	25
The Inhibition of Liver Alcohol Dehydrogenase by Thiols								
<u>Chapter 4</u>	42
The AMP Binding Site in Liver Alcohol Dehydrogenase								
<u>Chapter 5</u>	45
Chemical Modification of the Lysines of Liver Alcohol Dehydrogenase by Pyridoxal-5'-Phosphate								
<u>Chapter 6</u>	72
The Reaction of Methyl Picolinimide and 2-Methoxy-5-Nitro tropone with the Lysine Residues of LADH								
<u>Chapter 7</u>	86
Kinetic Studies on the Binding of Iodoacetate and other Anions to Liver Alcohol Dehydrogenase								
<u>Chapter 8</u>	102
The Reaction of Diethylpyrocarbonate with Histidine in Liver Alcohol Dehydrogenase								
<u>Chapter 9</u>	113
General Discussion								
References	133
Appendix	147

Preface

I hereby declare that this thesis was composed by myself and describes my own work carried out in the Biochemistry Department of Edinburgh University from 1st October, 1967 to 31st March, 1972.

I am grateful to the Science Research Council for a Research Studentship from 1st October, 1967 to 31st September, 1970 and the University of Edinburgh for a Graduate Research Scholarship under Edinburgh University Fellowships and Bursaries scheme from 1st October, 1970 to 31st March, 1972. I would like to thank Professor R.B. Fisher for the provision of laboratory facilities and both he and Professor G.S. Boyd for their kindness and help at opportune moments. I am very grateful to my supervisor, Dr. J.S. McKinley-McKee, for his generosity in the provision of equipment and chemicals, for the time he made available for advice, discussion and practical help and for his friendship; Dr. C.H. Reynolds, for his friendship and many useful discussions; Mr. J. McGowan and Mr. G. Pettigrew for their help and patience; and last but not least my wife Moira who typed this Thesis.

David L Moni

17th June 1972

Summary

The binding of ligands to Liver Alcohol Dehydrogenase (E) has been studied by steady state kinetics of inhibition. The nature of the binding sites was investigated by chemical modification studies of specific amino acid residues.

Mercaptoethanol and ethanethiol were found to be potent inhibitors and inhibition was studied at pH 7.1 and pH 9.5. This was supplemented by spectrophotometric binding studies. At pH 7.1 only E.DPN⁺-thiol complexes were detected but at pH 9.5 E.DPNH-thiol complexes are also formed. Concave-down Lineweaver-Burk plots are a notable feature of the inhibition by thiols. In particular the plots with varied DPN⁺ increase markedly in curvature with increasing pH. It is suggested that subunit interactions may be induced by thiol binding, which give rise to these effects. In the active centre thiol may be covalently bound to the C.4 of the nicotinamide ring of DPN⁺ rather than to zinc.

From the pH dependence of AMP and adenosine binding it is concluded that, in the coenzyme, the phosphate group nearer adenosine is important for binding. The possibility that the binding site for this phosphate is a lysine residue was investigated. Lysine in the enzyme was modified by pyridoxal-5'-phosphate, which introduces a negative group and was found to inactivate the enzyme; methylpicolinimidate, which introduces a positive group and enhances enzymic activity (Plapp, 1969); 2-methoxy-5-nitropropone, which introduces a neutral group and was found to concomitantly activate and inactivate the enzyme. The effect of DPNH, AMP and known ligands on the changes in enzymic activity

produced by reaction with these reagents and the extent of their incorporation into the enzyme was studied. The binding of AMP was shown to be considerably weakened by picolinimidation of the enzyme. It is concluded that one lysine in the active centre is important, but not essential for the binding of the phosphate of the AMP moiety of the coenzyme and that the coenzyme can accommodate considerable steric interference to form a catalytically active binary complex.

Iodoacetate and aromatic carboxylic acids were found to bind competitively with both the coenzyme and ethanol, but not acetaldehyde. These inhibitors are found to compete with the coenzyme by binding at two sites simultaneously and, since multiple inhibition studies indicated that they bind competitively with AMP and adenosine and largely non-competitively with orthophenanthroline, one of these sites is concluded to be the 'active centre' lysine. The pH dependence of halide binding and the interaction of chloride with other ligands was studied.

The role of histidine residues in the enzyme was investigated by studying the effects of modification of the enzyme with diethylpyrocarbonate. Diethylpyrocarbonate modifies 4 histidine residues per subunit of native enzyme and these residues are not protected by DPNH or DPNH plus isobutyramide. This modification results in a rapid enhancement of enzyme activity, which none of the ligands investigated protected against, followed by a slower inactivation process which leaves a residual activity. This inactivation process does not result in loss of coenzyme

binding capacity and is prevented by DPNH binding. The effect of other ligands on this inactivation process was studied. The kinetics of inactivation partially inactivated carbethoxyl-enzyme by iodoacetate were found to be very similar to those of native enzyme. It was concluded that none of the histidine carbethoxylated are involved in binding substrate or coenzyme, catalysis, or activation of cysteine-46. However, the tertiary structure of the native enzyme is sensitive to modification of these histidines.

Abbreviations

The following non-standard abbreviations have been employed.

LADH	:	Liver alcohol dehydrogenase
E	:	Signifies one active centre of LADH
CM-LADH	:	LADH in which cysteine-46 has been carboxymethylated with iodoacetate
O	:	Diphosphopyridine nucleotide (DPN ⁺)
R	:	Reduced diphosphopyridine nucleotide (DPNH)
Alc.	:	Alcohol
Ald.	:	Aldehyde
IB	:	Isobutyramide
ME	:	2-Mercaptoethanol
PLP	:	Pyridoxal-5'-phosphate
MP1	:	Methylpicolinimide
MNT	:	2-Methoxy-5-nitrotropone
BPCA	:	4-Biphenylcarboxylic acid
ØA	:	Phenylacetic acid
DEP	:	Diethylpyrocarbonate

CHAPTER 1.

Introduction.

Preface

Horse liver alcohol dehydrogenase was first crystallised by Bonnichsen and Wassen (1948) and has been extensively studied ever since. This chapter describes in outline the important discoveries arising from this work. Additional and more detailed information can be found in reviews by Theorell (1958), Sund and Theorell (1963), McKinley-McKee (1964) and Theorell (1964, 1967, 1970).

Molecular Structure

The method of purification has been improved by various workers (Bonnichsen and Brink, 1955; Dalziel, 1958a; 1961; Taniguchi, Theorell and Åkeson, 1967). These workers were faced with the problem of removing enzymically-active subfractions and electrophoresis of enzyme preparations by McKinley-McKee and Moss (1965) also showed that they contained several ethanol-active components.

Subsequent work has demonstrated the existence of two different subunit types, E and S (Pietruszko, Clark, Graves and Ringold, 1966; Theorell, Taniguchi, Åkeson and Skursky, 1966; Pietruszko et al., 1969; Pietruszko and Theorell, 1969). The S-type subunit has high activity with 3β - hydroxy - 5β steroids as substrates but little activity with ethanol, whereas the E-type subunit has no steroid dehydrogenase activity, but high ethanol activity. The S-type subunit is more basic than the E-subunit and on electrophoresis of LADH this gives rise to three main isoenzymes EE, ES and SS. In

addition, on electrophoresis there are minor bands migrating at positions more acidic to each of the main bands and these have been designated EE', EE" etc. According to Lutstorf and Wartburg (1969) EE' and EE" are interconvertible and they propose that this is because the subunits can exist in several conformational states. The existence of twelve enzymically active fractions has now been demonstrated (Lutstorf et al., 1970). The isoenzyme crystallised by Bonnichsen and Wassen (1948) was EE. ES (Theorell et al., 1966) and SS (Åkeson and Lundqvist, 1970) have also been crystallised.

The molecular weight of LADH was determined by Ehrenberg and Dalziel (1957, 1958) to be 83,000. More recent determinations by ultracentrifugational methods have given a molecular weight of 80,000. (Drum et al., 1967; Green and McKay, 1969). As described above the enzyme is a dimer and has been shown to dissociate into monomers of molecular weight 40,000. (Blomquist et al., 1967; Green and McKay, 1969; Butler, Jörnvall and Harris, 1969). X-ray crystallography indicates two symmetrically related halves per enzyme molecule (Brändén, 1965). There are two coenzyme binding sites per molecule of enzyme (Theorell and Bonnichsen, 1951; Theorell and Yonetani, 1963).

Amino acid sequence work has shown that the enzyme consists of two identical polypeptide chains and the active sequence has been elucidated (Jörnvall and Harris, 1970; Jörnvall, 1970 a, b, c). Each chain contains 374

amino acids, the N-terminal amino acid is an N-acetylserine and out of the seven histidines per chain six are located in the N-terminal half which is also rich in thiol groups. The molecular weight calculated from the chemical constituents is 79,955, when all species are in an uncharged form, which agrees closely with the physically measured molecular weight. The amino acid sequence of the S-subunit has also been worked out by Jörnvall (1970 d). This differs from the E-peptide in six positions five of which are compatible with one base mutations. The majority of the mutations occur in the N-terminal end part of the chain.

The thiols of LADH are the only amino acid residues of which the reactivity and function has been studied to any great extent. Parachloromercuribenzoate and derivatives of N-ethylmaleimide (Witter, 1960), Iodine (Li and Vallee, 1965), and silver ions (Bonnichsen, 1953; Wallenfels et al., 1959) all react fairly extensively. Iodoacetate, however, reacts specifically with one thiol per subunit (Li and Vallee, 1963, 1964; Harris, 1964; Evans and Rabin, 1968) now shown to be amino acid 46 in the sequence (Jörnvall, 1970 c).

Coenzyme and other ligands were shown to protect the enzyme from inactivation by iodoacetate and this thiol was therefore thought to be at the active centre. However, Reynolds and McKinley-McKee (1970 b) have shown that carboxymethyl enzyme retains 2-2.5% activity and forms the same binary and ternary complexes as native enzyme with the exception of stable highly fluorescent

complexes with DPNH and isobutyramide. These workers suggest that this thiol is not essential for activity or coenzyme binding, is not necessarily at the active site and may be concerned with conformational changes during the reaction cycle.

The functions of lysine and histidine residues in LADH is considered in later chapters.

LADH is a zinc metalloenzyme (Theorell et al., 1955; Vallee and Hoch, 1957) containing four zinc atoms per molecule (Åkeson, 1964; Oppenheimer et al., 1967; Drum et al., 1967 and Drum, Li and Vallee, 1969 a, b).

The following methods have been used to study the chemistry and function of zinc in LADH.

(a) The rate of exchange of zinc with ^{65}Zn in solution has been measured. (Druyan and Vallee, 1964; Drum et al., 1967; Drum et al., 1969 b). Coenzyme, substrates and other ligands retard the rate of exchange. Results suggest that subunits contain two zinc atoms which are in different environments. The removal of one correlates with loss of enzyme activity and is thought to be essential for catalytic activity (active centre zinc) whereas the other is less accessible and has been proposed to be necessary for maintaining enzyme structure (structural zinc).

(b) The effect on the enzyme of zinc chelating compounds. Spectral changes indicate that

orthophenanthroline (Vallee and Coombs, 1959) and 2,2'-bipyridyl (Sigman, 1967) are chelating a zinc atom when bound to the enzyme.

- (c) The properties of the zinc free enzyme have been studied (Hoagstrom et al., 1969) and DPNH binding has been shown to be preserved.
- (d) The zinc atoms of the enzyme have been replaced by cadmium or cobalt. Drum (1970) obtained derivatives which were active and in which all the zinc atoms had been replaced by cadmium or cobalt. Young and Wang (1971) have prepared a hybrid enzyme in which only the two zinc atoms thought to serve a catalytic function were replaced by cobalt.

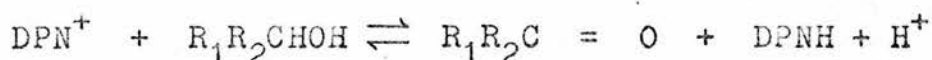
The effect of isotope substitutions in the reaction of DPNH with acetaldehyde catalysed by LADH (Mahler, Baker and Shiner, 1962; Baker, 1962; Palm, 1966 a, b) has indicated a transition state for hydrogen transfer similar to that encountered in the Meerwein-Ponndorf-Oppenauer equilibrium in which zinc plays an important role (Woodward, Wendler and Brutschy, 1945).

Zinc has been implicated in many theories of the active centre of LADH and has been proposed to bind substrate and the nicotinamide of the coenzyme. (Theorell et al., 1955; Theorell and McKinley-McKee, 1961 b, c; Plane and Theorell, 1961; Dalziel, 1963 a; Yonetani, 1963 a, b; Theorell and Yonetani, 1963;

Taniguchi et al., 1967; Sigman, 1967; Evans and Rabin, 1968).

Substrate Specificity

LADH catalyses the following reaction.



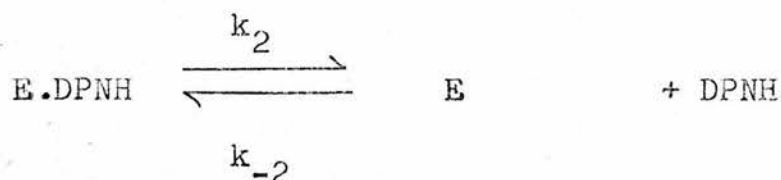
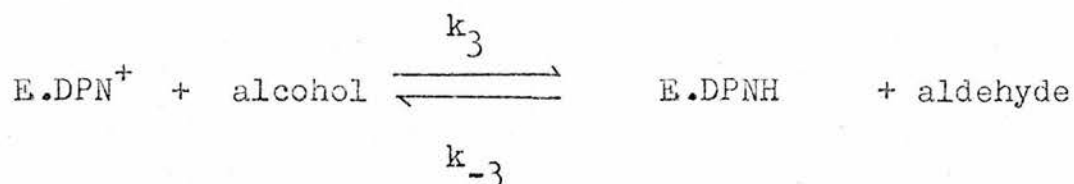
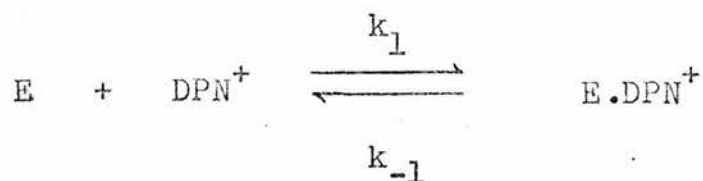
The enzyme exhibits a broad specificity and many primary and secondary alcohols and many aldehydes and ketones may be utilised as substrates (Sund and Theorell, 1963). Recently methanol has been shown to be a substrate (Mani et al., 1970).

The specificity of enzyme for coenzyme is not so broad as in the case of the substrates but a large number of coenzyme derivatives have activity (Theorell and Sund, 1963). The enzyme, however, is only active with the β -configuration of the coenzyme. TPN^+ exhibits a very weak activity (Dalziel and Dickinson, 1965 a). The enzyme specificity is for the A side of DPN^+ with a variety of substrates (Pópjak, 1970).

LADH also shows aldehyde mutase activity with acetaldehyde or formaldehyde as substrate (Abeles and Lee, 1960).

Theorell and Chance (1951) studied the rate of combination of LADH with DPNH by rapid flow experiments. They proposed the following mechanism in which they assumed that coenzyme must bind to the enzyme before substrate, that substrate must be released from the ternary complex before coenzyme and that the ternary complexes if they exist interconvert rapidly enough to

have no effect on the maximum velocity.

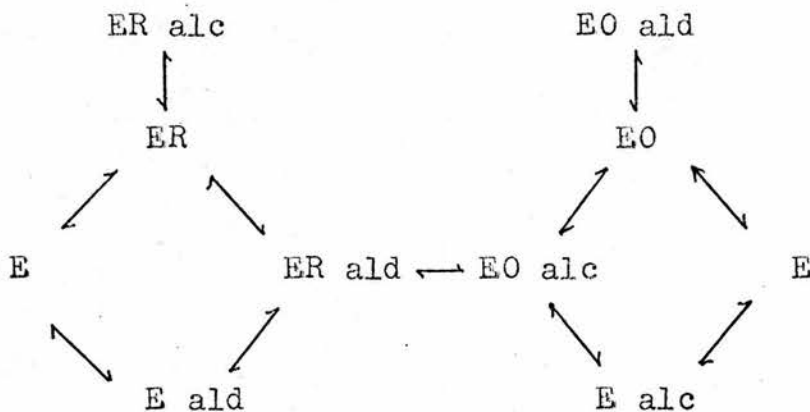


Thus at high coenzyme and substrate concentrations the rate limiting step is the release of coenzyme - product from the enzyme. It was shown by Theorell and Bonnichsen (1951) that binary complexes of enzyme and DPNH exist.

This mechanism now known as the Theorell-Chance mechanism was substantially supported by subsequent kinetic studies between pH 6.0 and 9.0. (Theorell et al., 1955; Theorell and McKinley-McKee, 1961 a, b, c; Dalziel, 1963 a, b). However, the existence of binary complexes of enzyme-alcohol and enzyme-aldehyde was demonstrated (Theorell and McKinley-McKee, 1961 a, b, c). Wratten and Cleland using the sensitive method of product inhibition (1963), and alternate product

inhibition (1965), proved the existence of ternary complexes. Silverstein and Boyer (1964) used radioactive tracers to follow the interconversion of coenzymes and substrates under equilibrium conditions. They concluded that coenzyme is preferentially bound before substrate but that 1-2% of the reaction takes place by the alternative pathway. Ethanol causes high substrate inhibition by forming an "abortive" enzyme-DPNH-ethanol complex, which releases DPNH more slowly than enzyme-DPNH. (Theorell et al., 1955; Theorell and McKinley-McKee, 1961 c; Dalziel and Dickinson 1966 a, b; Shore and Theorell, 1966; Wratten and Cleland, 1965).

The mechanism below in which the upper pathway is highly preferred is a modification of the Theorell-Chance mechanism which incorporates these subsequent discoveries.



Some studies on the pre-steady state kinetics of the LADH mechanism have been made by rapid reaction techniques. (Theorell and Chance, 1951; Geraci and Gibson, 1967; Shore, 1969; Theorell et al., 1970).

These studies all indicate that coenzyme and substrate binding to the two sites per molecule is independent and identical. However, Bernhard et al., (1970) studying the transient kinetics of the reduction of aromatic aldehydes showed that the rate of formation of enzyme-DPNH-aldehyde was the same for each site but that the rate of catalysis was different. Dutler (1970) has shown that this result depends on the substrates and buffers used.

Shore and Gutfreund (1970) identified a step representing an isomerisation of enzyme-DPNH which was rate determining in the presence of chloride and identified a step which is either the hydride transfer or is controlled by the hydride transfer rate.

Ligand Binding and Inhibition

Although all the inhibitors do not fall neatly into the following categories these provide some order in a discussion of the variety of compounds which inhibit LADH. This section deals mainly with ligands which have been employed in the studies described in the following chapters.

- (a) Fatty acids form ternary complexes with enzyme and DPN^+ (Winer and Theorell, 1960) and have been considered to bind in an analogous manner to alcohol. They also form binary complexes with enzyme but do not bind to enzyme-DPNH. Iodoacetate inactivates the enzyme but also binds reversibly (Reynolds and McKinley-McKee, 1969; Reynolds et al., 1970).

- (b) Fatty acid amides (Winer and Theorell, 1960; Theorell and McKinley-McKee, 1961 b, c; Woronick, 1961, 1963 a, b) form ternary complexes with enzyme-DPNH. They are considered to bind as aldehyde analogues. It has been shown, however, that they can form ternary complexes with enzyme-DPN⁺ (Sigman and Winer, 1970).
- (c) Coenzyme fragments adenine nucleotides compete with both coenzymes for the enzyme. (Theorell and Yonetani, 1964).
- (d) Various aromatic acids: 4.biphenyl carboxylic acid and phenanthroic acid (Sigman, 1967), salicylic acid (Dawkins et al., 1967), various thyroxine derivatives (McCarthy et al., 1968; McCarthy and Lovenberg, 1969; Gilleland and Shore, 1969) have been shown to be competitive with coenzyme but none have been demonstrated to form a ternary complex with enzyme-DPN⁺. Also, fatty acids form binary complexes with enzyme by binding to zinc (Sigman, 1967), whereas aromatic acids do not (Sigman, 1967; Gilleland and Shore, 1969).
- (e) Zinc binding compounds Orthophenanthroline and 2,2'-bipyridyl have been mentioned previously. Sigman (1967) used the spectral changes occurring on the binding of 2,2'-bipyridyl to the enzyme to investigate the binding of a large range of substrates and inhibitors. Pyrazole

forms a ternary complex with enzyme-DPN⁺ (Theorell and Yonetani, 1963). It is thought to form an adduct with the pyridine ring of DPN⁺. Theorell et al., (1969) have surveyed the inhibition of the enzyme by a large variety of monodentate nitrogen bases. Imidazole forms binary complexes and ternary complexes with both enzyme-DPN⁺ and enzyme-DPNH (Theorell and McKinley-McKee, 1961 b, c). A remarkable phenomenon is the rate enhancement at high coenzyme and substrate concentrations due to enzyme-DPNH-imidazole releasing DPNH more rapidly than enzyme-DPNH and inhibition at low substrate concentration. This phenomenon also occurs with 2,2'-bipyridyl (Sigman, 1967) and some halides (Theorell et al., 1955). Thiols are capable of forming strong sulphur bonds with zinc and have been observed to form ternary complexes with enzyme-DPN⁺ (Van Eys et al., 1958).

- (f) A variety of simple anions bind to the enzyme (Theorell et al., 1955; Plane and Theorell, 1961). They are often competitive with both coenzyme and ethanol. Halide binding has been followed by the line broadening of NMR spectra of ³⁵Cl⁻ (Zeppezauer et al., 1969). The anionic dyes Rose Bengal and 8-anilino-naphthalene sulphonate (Brand, Gohlke and Kao, 1967; Turner and Brand, 1968) bind to the

enzyme and the enhancement of their fluorescence produced was used to measure their binding. They probably bind in a similar fashion to aromatic acids. Auramine O is a fluorescent cationic dye and unlike anionic dyes is not competitive with coenzyme and may have a binding site distinct from many of the other known inhibitors (Conrad, Heitz and Brand, 1970).

CHAPTER 2.

Materials and Methods.

Liver Alcohol Dehydrogenase (E.C. 1.1.1.1.)

The enzyme was purchased from C.F. Boehringer and Soehne (Mannheim), who had prepared it by the method of Dalziel (1958). It consisted to a large extent of the form EE. The enzyme was stored as a crystalline suspension (8 mg/ml) in 30% ethanol at -14°C . To prepare for use, crystals were collected from an appropriate volume of the suspension by centrifuging at 15,000 r.p.m., dissolved in glycine NaOH buffer (pH 10), and dialysed for 3 days at $0-4^{\circ}\text{C}$ in Visking dialysis tubing against 2 litres of 40mM phosphate buffer (ionic strength, 0.1, pH 7.4). The buffer was changed twice. The dialysis tubing was soaked overnight in distilled water before use. After dialysis the enzyme solution was centrifuged to remove any precipitated denatured protein.

The protein concentration (μM) was determined from its absorbance at 280nm assuming an absorbance of 0.455 for a 1 mg per ml solution (Bonnichsen, 1950; Taniguchi et al., 1967). The molecular weight of the protein was taken as 84,000 (Ehrenberg and Dalziel, 1957, 1958).

The catalytic activity of the enzyme was determined using the following assay (Dalziel, 1957). 1.0 ml, DPN^{+} (1mg/ml); 0.15 ml. ethanol; 0.1M glycine NaOH buffer (pH 10), 1.85 ml; equilibrated at 23.5°C . A small amount of enzyme solution (V ml) was stirred in rapidly on a glass stirring rod and the time required for the absorbance at 340nm to increase by 0.2 ($t_{0.2}$) was measured. The active enzyme concentration is obtained from the following formula.

$$\text{Enzyme } (\mu\text{N}/_2) = \frac{1.13}{t_{0.2.V}} \times \frac{1}{84,000} \times \frac{3.00+V}{3.00} \times 0.83$$

The enzyme concentration is inversely proportioned to $t_{0.2}$ and the first term gives the enzyme concentration in mg/ml (Dalziel, 1958). The factor 0.83 is the ratio of the binding site concentration obtained by fluorescence titration with DPNH in the presence of excess isobutyramide to the enzyme concentration obtained from the first three terms in the above formula (Theorell and McKinley-McKee, 1961 b). Compared with the protein concentration determined from absorbance at 280nm, activity gave an apparent purity of 83-94%. Reynolds (1970), using the same samples, found that the binding sites concentration (measured by fluorescent titration with DPNH in the presence of excess isobutyramide, or by titration with DPN^+ in the presence of pyrazole) gave an apparent purity of 90-100%.

Preparation of carboxymethylated-LADH (Reynolds and McKinley-McKee, 1970)

Native enzyme was alkylated with iodoacetate (4-6mM) at room temperature in the presence of imidazole (1-4mM). After 1 to 2 hours, when the remaining activity was 2-2.5%, imidazole, iodoacetate and iodide were removed by passage through a column (1.4 x 12cm) of Sephadex G.25 (medium). This procedure specifically carboxymethylates one thiol (cysteine 46) per subunit of the enzyme.

Coenzymes

DPN⁺ was obtained from Sigma Chemical Co. (St.

Louis), C.F. Boehringer and Soehne (Mannheim), and P.-L. Biochemicals (Milwaukee). The DPN^+ , chromatopure grade, obtained from P.-L. Biochemicals was found to contain one mole of alcohol per five moles of DPN^+ and was thus only used when high ethanol concentrations were present. DPN^+ was dissolved in distilled water, giving an acidic solution (pH 3.4) and the concentration was determined from the absorbance at 260nm at pH 7.1 (Millimolar extinction coefficient = 18; Kornberg and Pricer, 1953; Siegal, Montgomery and Bock, 1959). Such solutions of DPN^+ are stable for many days when kept in the dark at 2°C . The proportion of DPN^+ measured at 260nm which was enzymically reducible was obtained by measuring fluorimetrically the amount of DPNH produced when the DPN^+ was reduced by excess ethanol and LADH at pH 10. On this basis the samples contained 96% of reducible DPN^+ .

DPNH was obtained from Sigma Chemical Co. (St. Louis) and from P. -L. Biochemicals (Milwaukee). Solutions were made in 0.01 M Tris (hydroxymethyl) amino methane (pH about 8.5) and the concentration was determined from the absorbance at 340nm at pH 7.1. (Millimolar extinction coefficient = 6.25; Rafter and Colowick, 1957). Solutions of DPNH were prepared and used within 24 hours. They were kept in the dark at 2°C when not in use. The amount of DPNH oxidised by acetaldehyde and LADH was determined fluorimetrically at pH 7. For the samples used the fluorescence was reduced to 2% of the original value.

The dry coenzyme samples were stored in desiccators

at -14°C . Other chemicals were of the purest commercially obtainable grades.

Adenosine was obtained from Sigma Chemical Co. (St. Louis). AMP and ADP-ribose were obtained from P. -L. Biochemicals (Milwaukee). The concentrations of solutions of these compounds were determined from the absorbance at 260nm at pH 7 using the millimolar extinction coefficient for adenine of 15.4 (Morell and Bock; 1954). Pyridoxal-5'-phosphate was obtained from P. -L. Biochemicals and solutions were made by weighing the solid out accurately, dissolving in distilled water and titrating to the desired pH with 2N NaOH. 2-Methoxy-5-nitrotropone was obtained from Sankyo & Co. (Tokyo).

Thiols were obtained from Fluka AG Buchs S.G. The concentration of solutions in distilled water was determined using 5,5'-dithiobis-(2-nitrobenzoic acid). With excess of the reagent thiophenylate ion is liberated by disulphide exchange with the thiol to give a concentration of thiophenylate ion equal to the thiol concentration. The concentration of thiophenylate is calculated from the absorbance at 412nm using a millimolar extinction coefficient of 13.6 at pH 7 (Ellman, 1959). Stock solutions of thiols ($>1\text{mM}$) were stable for several hours. Dithiothreitol was added to stabilize more dilute solutions.

A few chemicals were further purified before use. Iodoacetate was re-crystallised by Dr. J.S. McKinley-

McKee or C.H. Reynolds) from 60° - 80° petroleum ether. It was white with a melting point $81.1 - 81.7^{\circ}\text{C}$, and iodine content 68.26% (Reynolds, 1970). Solutions of iodoacetate in distilled water were neutralised to just below pH 5 by addition of 2N NaOH. At pH values higher than pH 5 iodoacetate hydrolyses.

Imidazole and isobutyramide were freed of fluorescent impurities by treatment with charcoal and recrystallised by Dr. J.S. McKinley-McKee.

4-Biphenylcarboxylic acid (Fluka AG, Buchs S.G.) was recrystallised from methanol and methanol-water by Dr. J.S. McKinley-McKee. 4-Biphenylcarboxylic acid was dissolved in distilled water by addition of a few drops of 2N NaOH.

N-Ethylmorpholine (BDH) and acetaldehyde (Fluka AG, Buchs S.G.) were distilled immediately before use.

Distilled water

Tap water was distilled in a Manesty still and re-distilled from dilute alkaline permanganate the water being condensed in quartz and the first litre collected being discarded. This water was used for making all solutions.

Glassware

Carlsburg micropipettes ($3-500\ \mu\text{l}$) were used. They were calibrated from the absorption at 350nm of the amount of sodium dichromate they delivered using the method of equal dilutions with calibrated volumetric flasks. They were cleaned by filling with chromic acid, washing and soaking with tap water, washing with distilled water and

sucking air through till dry.

Other glassware and quartz cuvettes were cleaned by soaking in chromic acid, 50% nitric acid or a 5% Decon 65 solution. They were then thoroughly rinsed with hot water, allowed to soak in water washed several times with distilled water and oven dried at 70°C. Volumetric glassware was allowed to drain dry.

Instruments

pH measurements were made on a Radiometer pH meter (model pH 26). A glass electrode and KCl electrode were used.

A Hilger Uvispek spectrophotometer or a Gilford Model 2000 recording spectrophotometer were used for spectrophotometric measurements. Both had cuvette chambers thermostatted at 23.5°C.

Amino acid analysis was performed by the accelerated method of Spackman, Moore and Stein (1958), using a Locarte automatic amino acid analyser with the assistance of Mr. Jack McGowan.

A Stanton balance (model C.L 41) capable of measuring to four decimal places was used for most weighings, whereas a Stanton balance (model MC5) capable of measuring to five decimal places was used for weighings when only a few mgs of sample were required.

A Beckmann DK2 recording spectrophotometer was used for measuring difference spectra.

A Farrand recording spectrofluorimeter was used for measuring fluorimetric titrations.

The Fluorimetric Measurement of Steady State Kinetics

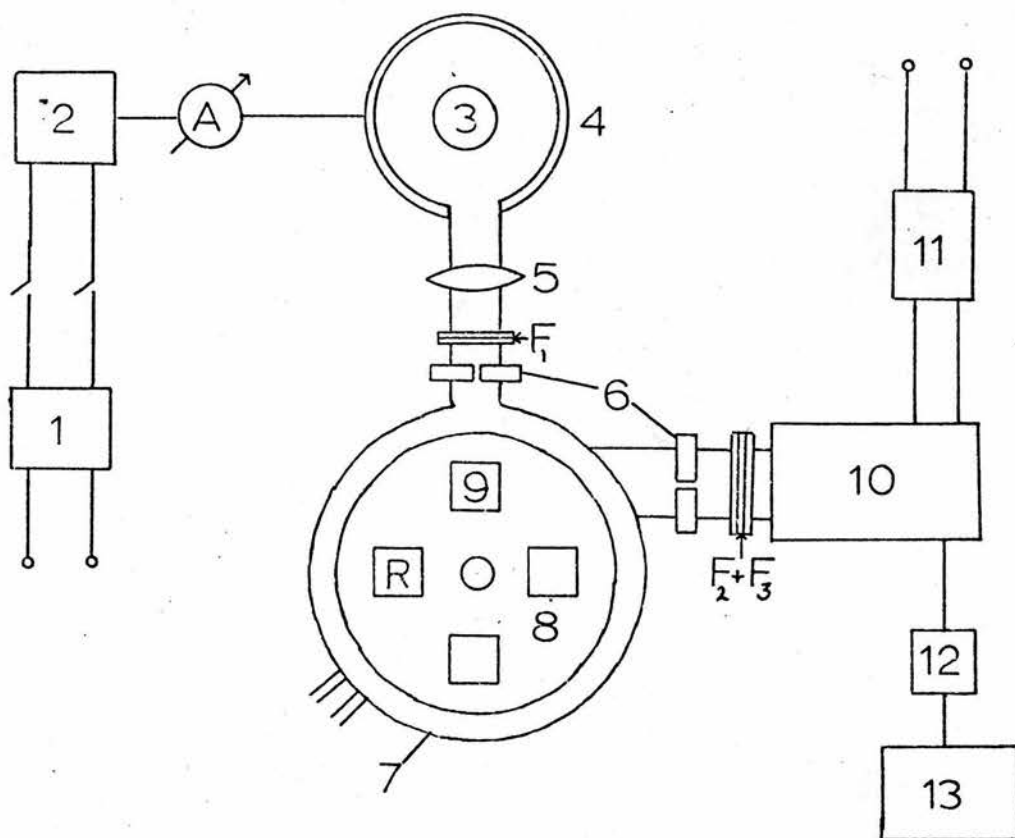


Fig.2-1. The Recording Fluorimeter. The lay-out of this instrument is fully explained in the text. The design of this fluorimeter is similar to that of instruments developed by Theorell and Nygaard (1954) and Dalziel (1962).

The rate of LADH catalysed oxidation of ethanol by DPN^+ or reduction of acetaldehyde by DPNH was followed by observing the appearance or disappearance respectively of DPNH fluorescence in a recording fluorimeter.

The Recording Fluorimeter

The lay-out of this instrument is shown in fig. 2-1 and explained below. The light source (3) is a mercury lamp (Osram Wotan Hg/2), which is enclosed in a water cooled jacket (4). The power supply to the lamp is from the mains and a constant voltage transformer (1). A variable inductance or choke (2) is necessary and a current of 1.1 - 1.2 amps was maintained for optimum lamp stability. The light is condensed by a glass lens (5) and passes through a Zeiss M366 filter (F_1) with a transmission maximum at 366nm and zero transmission above 400nm and below 340nm. Adjustable slits at position (6) determine the geometry of the excitation light path through the solution and the geometry of the emitted fluorescent light reaching the detector (10). The slits are 1cm high and the widths can be varied from 1mm to 8mm by 1mm increments. Fluorescent free glass cuvettes (1cm x 1cm x 4.5cm) are placed in holes (9) in a solid revolvable block (8) set in a thermostatted jacket (7) which was maintained at 23.5°C . A solution of DPNH(R) was used as a fluorescent reference standard and was kept permanently in position (R) during experiments. The other two cuvette positions were used to equilibrate solutions to the correct temperature. The fluorescent light passes through a Jena FG 10 filter (F_2) which absorbs scattered UV light and

then through a Jena GG 420 filter (F_3). This combination results in absorption of all light below 417nm and 60% transmission at 460nm. The detector (10) is an EMI 9656A photomultiplier which was selected for high sensitivity in the blue region and very low dark current. The photomultiplier is stabilised by a xenon dynode (IS 4150) across the first stage. The photomultiplier has a 44 mm aperture. A Keithley model 246 E.H.T. supply (11) provides the voltage for the photomultiplier. This voltage was kept within the range 800-1500 volts which is optimum for the photomultiplier. The current generated by the photomultiplier is measured by a Keithley model 414S picoammeter (12) and recorded by a Keithley model 370 recorder (13). Chart speeds of 1"/10 secs, 1"/20 secs. and 1"/40 secs. were used. The picoammeter enables high fluorescent signals to be "backed off" which is essential for the accurate measurement of initial rates when high sensitivity is used.

The Measurement of Initial Rates by Fluorimetry

The presence of debris particularly small fibres from filter paper or clothing caused large distortion of the recorder tracing when they floated into the light path, particularly at high sensitivity. Solutions were cleared by filtering through Sartorius or Millipore membrane filters with a 0.45 micron pore size, directly into volumetric flasks in an enclosed all glass system. The tips of pipettes were wiped with hard filter paper.

All cuvette solutions were made up to 4 ml. The temperature was 23.5°C through out. Enzyme solutions

were kept on ice and the reactions initiated by stirring an aliquot on the end of a glass stirring rod into the cuvette solution. Measurements were made in duplicate. At very slow initial velocity a greater number of determinations were sometimes necessary. The enzyme was assayed at the beginning and the end of experiments. If there was any significant loss of activity it was assumed to have occurred linearly over the time of the experiment and the rates were corrected. The pH of each cuvette solution was determined after each initial rate measurement.

Acetaldehyde was kept at -14°C and distilled on the evening before the experiment. Solutions were kept at 0°C and assayed on the day of the experiment by the oxidation of DPNH at pH 7.1 catalysed by LADH. Care was taken not to expose solutions to be used for measurement of the rate of oxidation of ethanol to acetaldehyde and vice versa. 95 volume % ethanol containing 5% water was used and stored at -14°C .

The enzyme concentration was adjusted so that the initial rate was constant for 30 secs. or more. The initial velocity (V_o) was given by,

$$\frac{e}{V_o} = \frac{e}{V_p \cdot S \cdot (R)} F$$

where e is the binding site enzyme concentration (μN);

F is the deflection of the DPNH standard in chart units at $S = 1$ and was measured after each rate determination; S is the sensitivity which was varied from 3 to 0.01;

(R) is the concentration of the fluorescence reference standard solution of DPNH (10-13 μ M): V_p is the recorder pen velocity in chart units per second. The initial rate data is presented as Lineweaver-Burk plots with the line drawn by eye.

The fluorescence of DPNH is quenched by increasing DPNH concentration. Calibration curves of the ratio of the fluorescence of DPNH to the fluorescence of the reference standard at the same sensitivity against DPNH concentration were determined for the different slit combinations which were used. The calibration curve for the slit combination (6mm and 6mm) is shown in fig. 2-2. The reference standard gave negligible quenching. The above slit combination was used in the experiments described.

CHAPTER 3.

The Inhibition of Liver Alcohol Dehydrogenase
by Thiols

Introduction

It is known that a variety of thiols structurally related to alcohol substrates form ternary complexes with LADH and DPN^+ (Van Eys et al., 1958). Sigman (1967) has also shown that ethanethiol and butanethiol form binary complexes with the enzyme at pH 7.0 by binding to the active centre zinc in the enzyme. A non-enzymic reaction also occurs between thiols and DPN^+ . This has been postulated by Van Eys and Kaplan (1957) to be addition of the thiol at carbon-4 of the nicotinamide ring, and by Kosower (1956) to be formation of a charge transfer complex between the thiol and the nicotinamide ring. By studying the steady state kinetics of LADH, in the presence of thiols, it was hoped to gain information on the nature of the above ternary complex and to investigate any other interactions of thiols with the enzyme which might be revealed. Since thiols are capable of forming strong sulphur bonds with zinc, these studies may indicate the role of zinc in ternary complexes of compounds with enzyme and coenzyme.

Mercaptoethanol was chosen for initial experiments since, it is highly soluble in water, relatively stable and structurally similar to ethanol. Although it is a substrate for the enzyme it has a slow turnover compared to ethanol (Lamb and Williams, 1965). The structural analogue, ethanethiol, as well as various other thiols were studied, as they are powerful inhibitors, but are not substrates.

Results

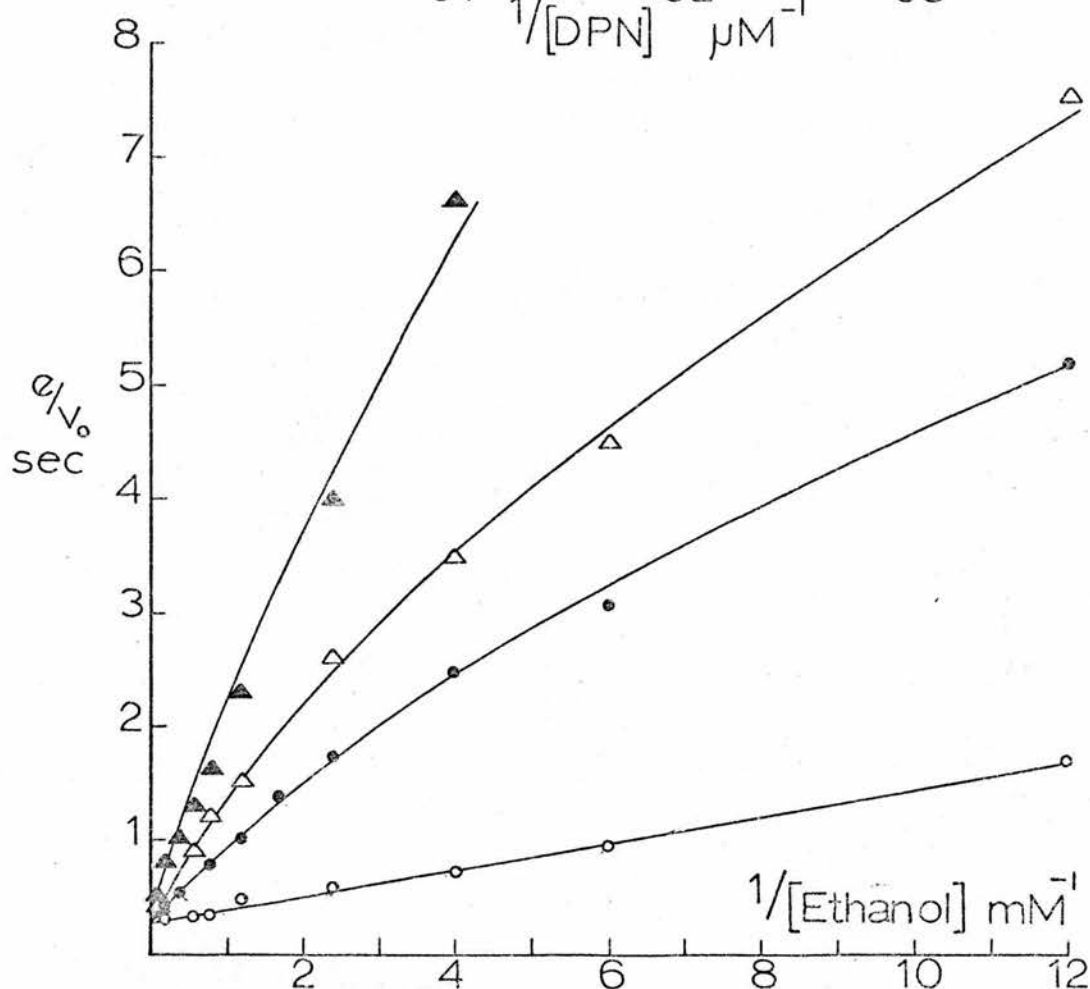
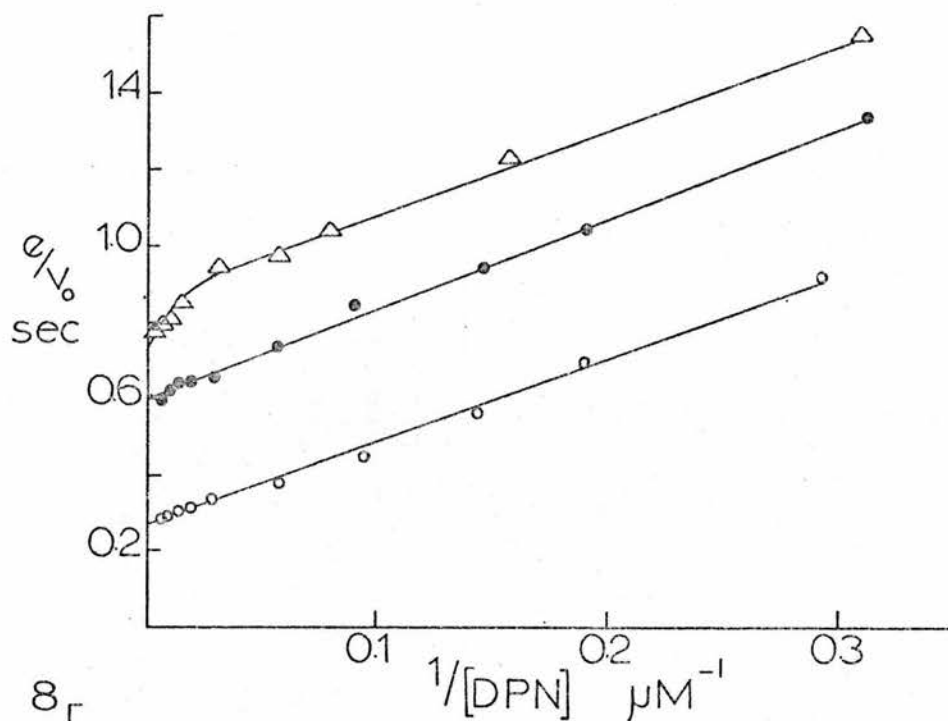


Fig.3-1. Kinetics in the presence of mercaptoethanol at constant ethanol concentration (8.25mM) and varied DPN^+ concentration (case-1). Phosphate buffer, pH 7.14, ionic strength 0.1; o, without inhibitor; + mercaptoethanol, •, (180 μM), Δ , (360 μM).

Fig.3-2. Kinetics in the presence of mercaptoethanol at constant DPN concentration (260 μM) and varied ethanol concentration (case-2). Phosphate buffer, pH 7.14, ionic strength 0.1; o, without inhibitor; + mercaptoethanol, •, (70 μM), Δ , (104 μM), \blacktriangle , (180 μM).

The inhibition by ME of the LADH catalysed reaction was studied in four types of experiment as below;

	Constant	Varied
Case-1	Ethanol	DPN ⁺
Case-2	DPN ⁺	Ethanol
Case-3	Acetaldehyde	DPNH
Case-4	DPNH	Acetaldehyde

Inhibition by ME at pH 7.14

Case-1 (fig. 3-1): The pattern of inhibition is uncompetitive. However, the parallel lines are asymptotes of curves which are concave-down near the ordinate. This effect is noticable only when the inhibition is greater than 50%.

Case-2 (fig. 3-2): ME is strictly competitive with ethanol. Inhibition thus seems to be caused by formation of EO.ME and ER.ME is not significant. The plots are concave-down because ME is a substrate. However, inhibition constants were obtained from the slopes of the tangents to the curves at the ordinate. These constants which are given in table 3-1 are not the true dissociation constants for the EO.ME complex ($K_{EO,I}$). This is because they depend on g , the ratio of the turnover number of ME to the turnover number of ethanol. The slope of the tangent is given by,

$$K_m/V_{\max} \left[1 + (ME)/K_I(1-g) \right] \quad :(\text{Reiner, 1969}).$$

TABLE 3-1

Inhibition constants for mercaptoethanol and ethanethiol at pH 7.14 and 9.5 calculated from the slopes of case-2 (figs. 3-2, 3-6, 3-10 and 3-12).

pH 7.14		pH 9.5	
ME	k_I	ME	k_I
μM	μM	μM	μM
70	11	18	13
104	11	36	17
180	11	72	19
		144	19
Average 11		Average 17	

Ethanethiol k_I		Ethanethiol k_I	
μM	μM	μM	μM
1.7	0.07	0.1	0.03
2.8	0.07	0.3	0.03
Average 0.07		Average 0.03	

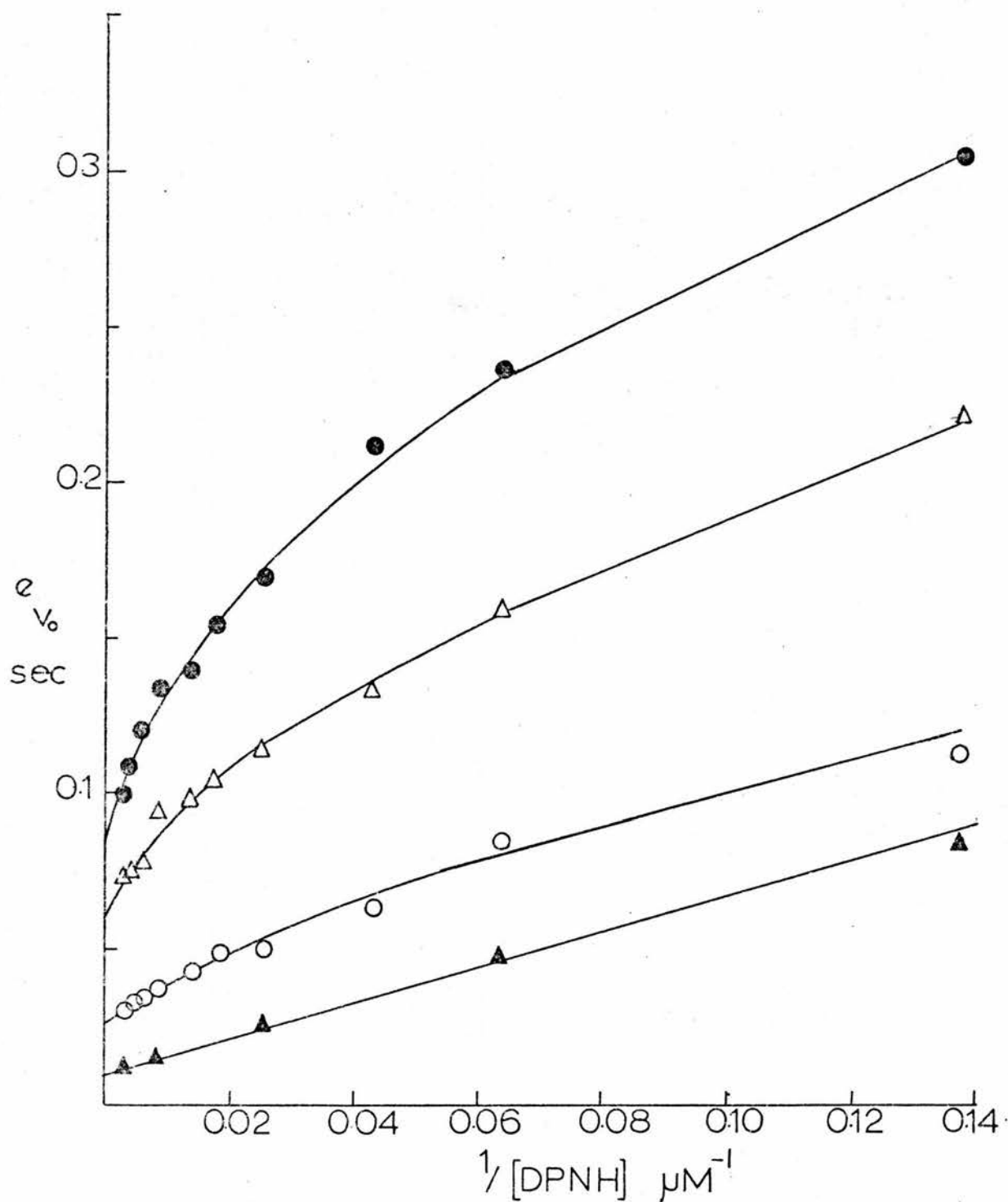


Fig.3-3. Kinetics in the presence of mercaptoethanol at constant acetaldehyde concentration (3.70mM) and varied DPNH concentration (case-3). Phosphate buffer, pH 7.14, ionic strength 0.1; ▲, without inhibitor; + mercaptoethanol, ○, (70 μM), Δ, (360 μM), ●, (710 μM).

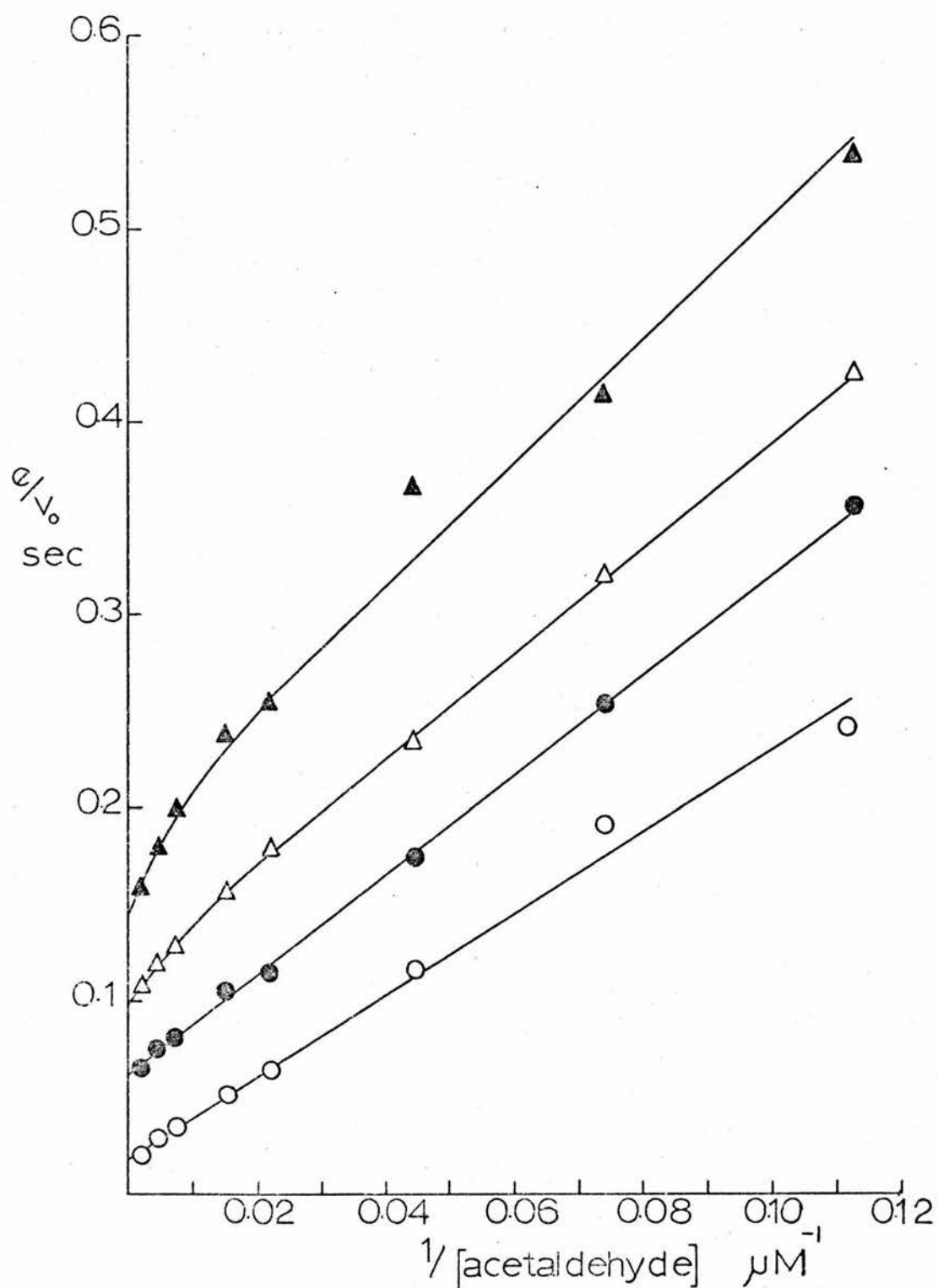
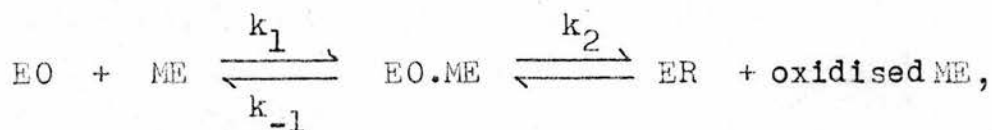


Fig.3-4. Kinetics in the presence of mercaptoethanol at constant DPNH concentration ($15.4 \mu\text{M}$) and varied acetaldehyde concentration (case-4). Phosphate buffer, pH 7.14, ionic strength 0.1; o, without inhibitor; + mercaptoethanol, •, ($140 \mu\text{M}$), Δ , ($360 \mu\text{M}$), \blacktriangle , ($710 \mu\text{M}$).

In the case of ME, g was measured to be about 0.03 at pH 7.14, so K_I is nearly the same as the K_I determined from the slopes. This K_I is still not the dissociation constant, $K_{EO,ME}$ since;



from which it follows that $K_I = (k_{-1} + k_2)/k_1$. The very low turnover number for ME may be due to the hydroxyl group and the thiol group of the molecule competing for the same binding site at the active centre. Thus a constant proportion of inhibition will always exist.

Case-3 (fig. 3-3): The plots are nearly parallel at low DPNH concentration, but are concave-down near the ordinate.

Case-4 (fig. 3-4): The plots are linear at low acetaldehyde concentration, but exhibit a slight concave-down curve near the ordinate. The lines are almost but not quite parallel.

Inhibition by mercaptoethanol at pH 9.5

The pK_a of the thiol group of ME has been reported to be 9.5 (Calvin, 1954), 9.444 (Antikaninen and Tevanen, 1962), 9.72 (Irving et al., 1964) and 9.51 (Lienhard and Jencks, 1966). Thus at pH 9.5 about half the ME is in the thiolate form.

Case-1 (fig. 3-5): Curves of the same form as in fig. 3-1 were obtained, but they are very much more pronounced.

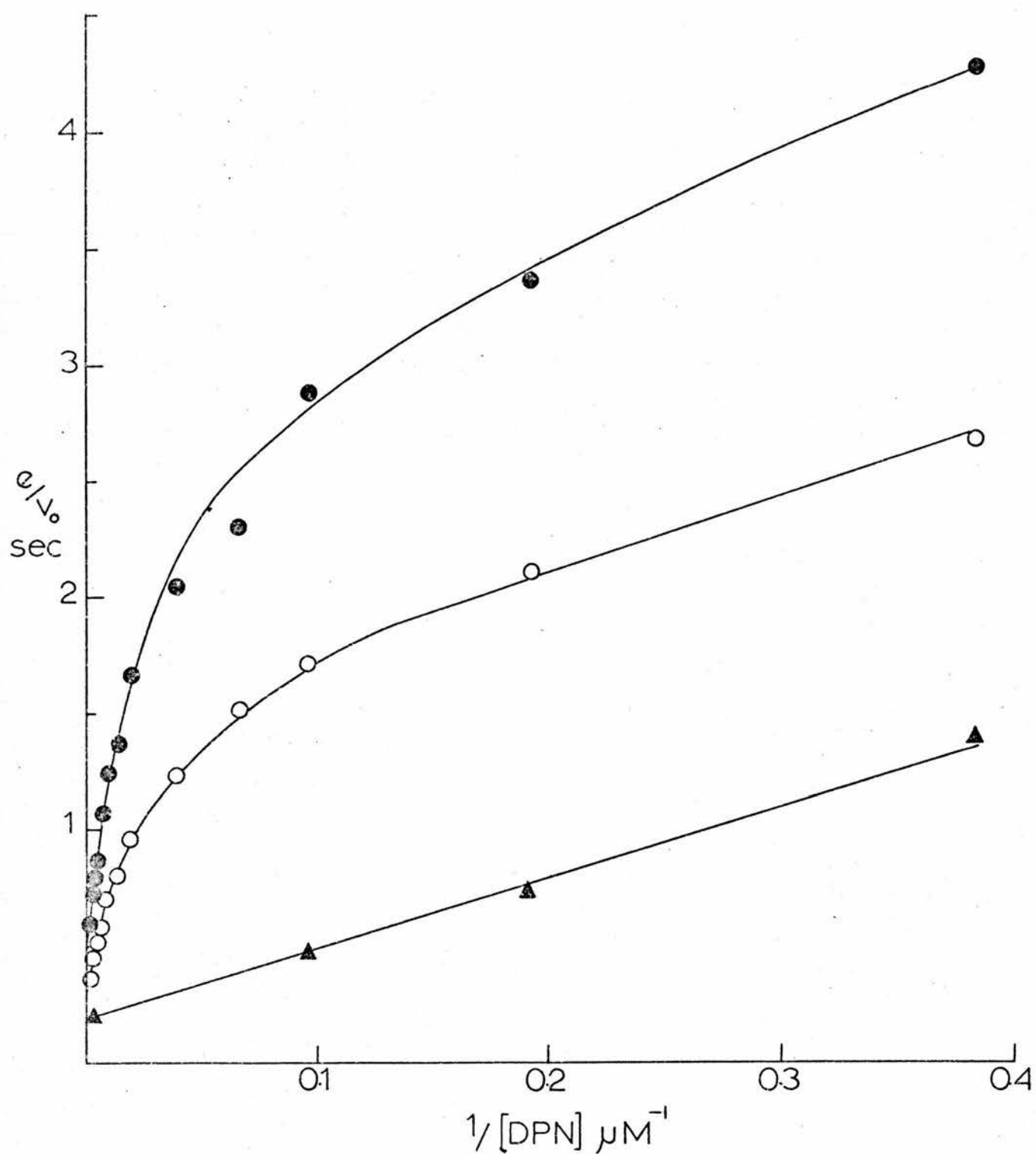


Fig. 3-5. Kinetics in the presence of mercaptoethanol at constant ethanol concentration (8.25mM) and varied DPN^+ concentration (case-1). 10mM glycine-NaOH, pH 9.5, plus phosphate to give an ionic strength of 0.1; ▲, without inhibitor; + mercaptoethanol, ○, (45 μM), ●, (90 μM).

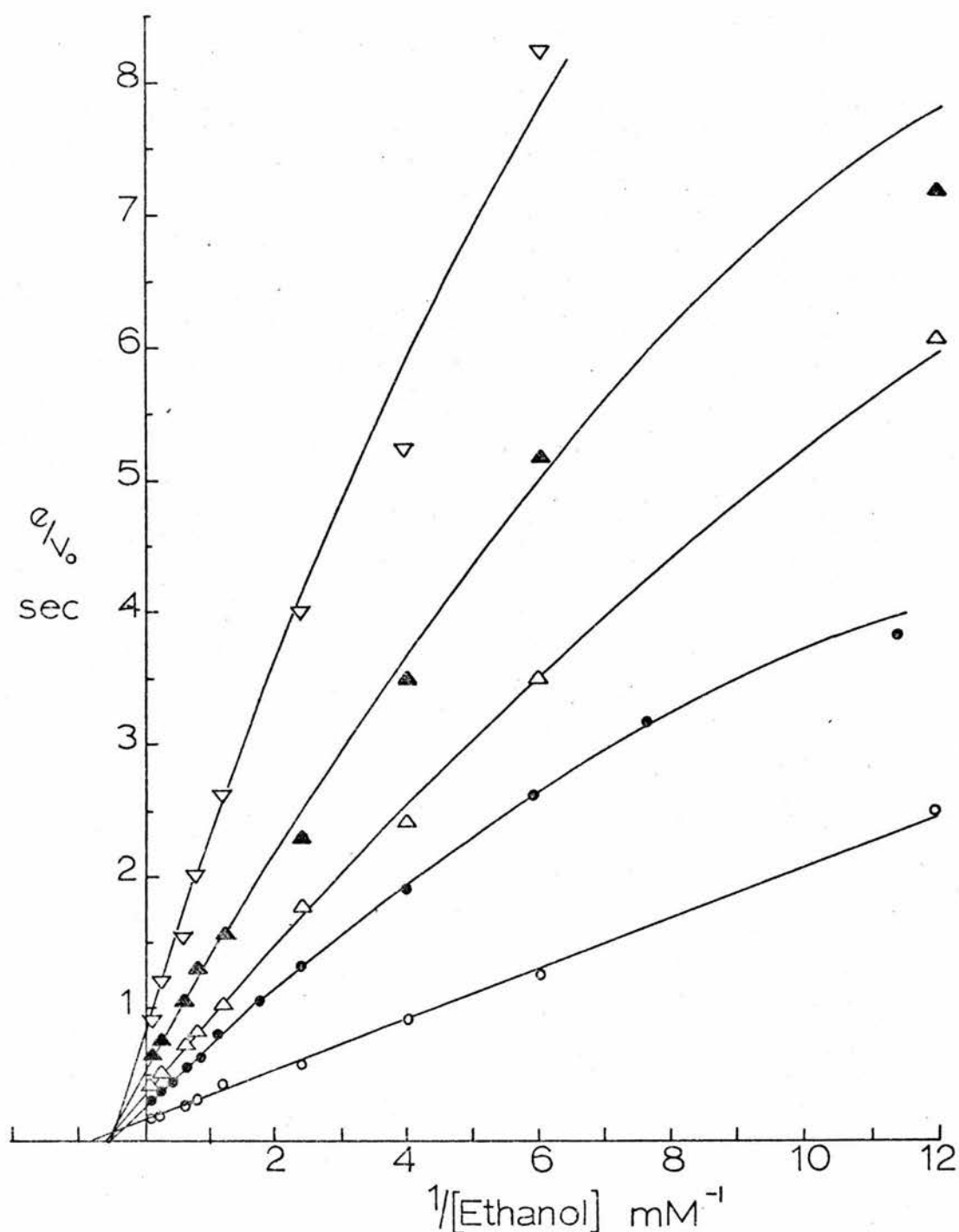
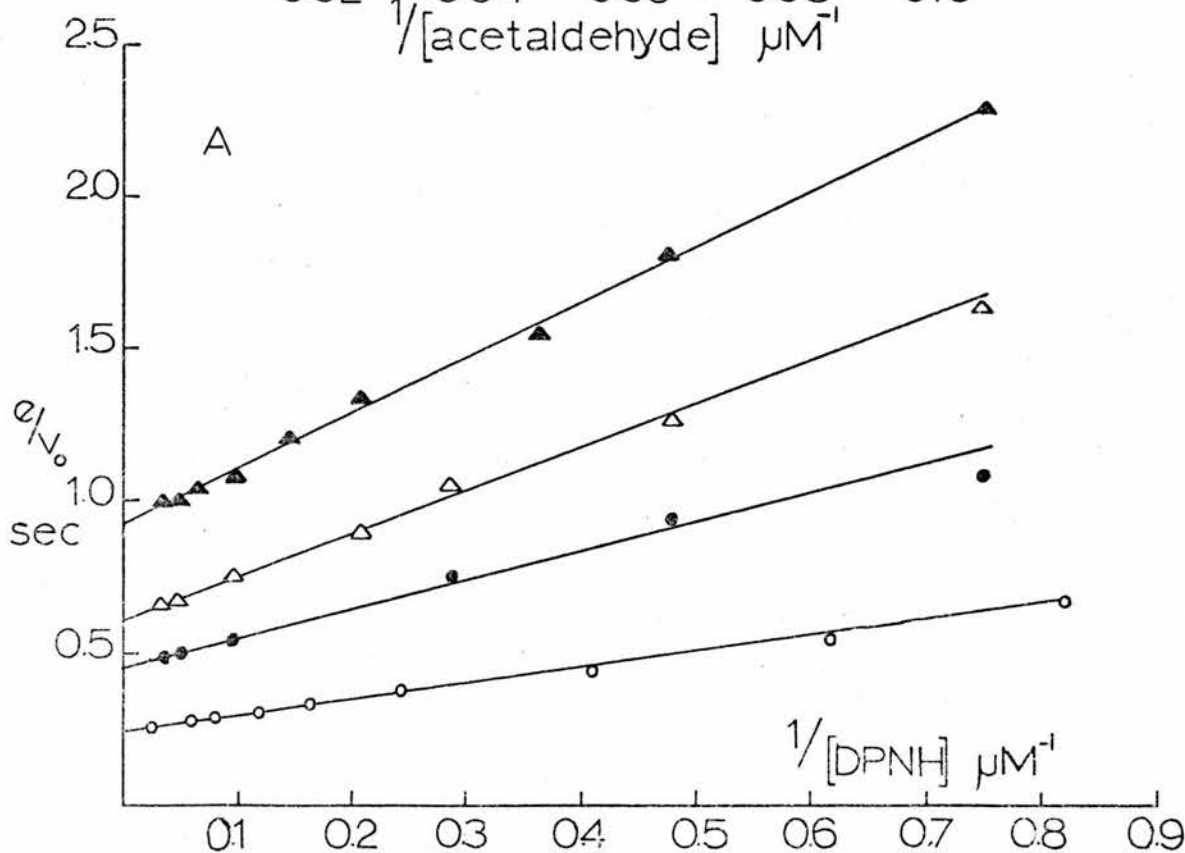
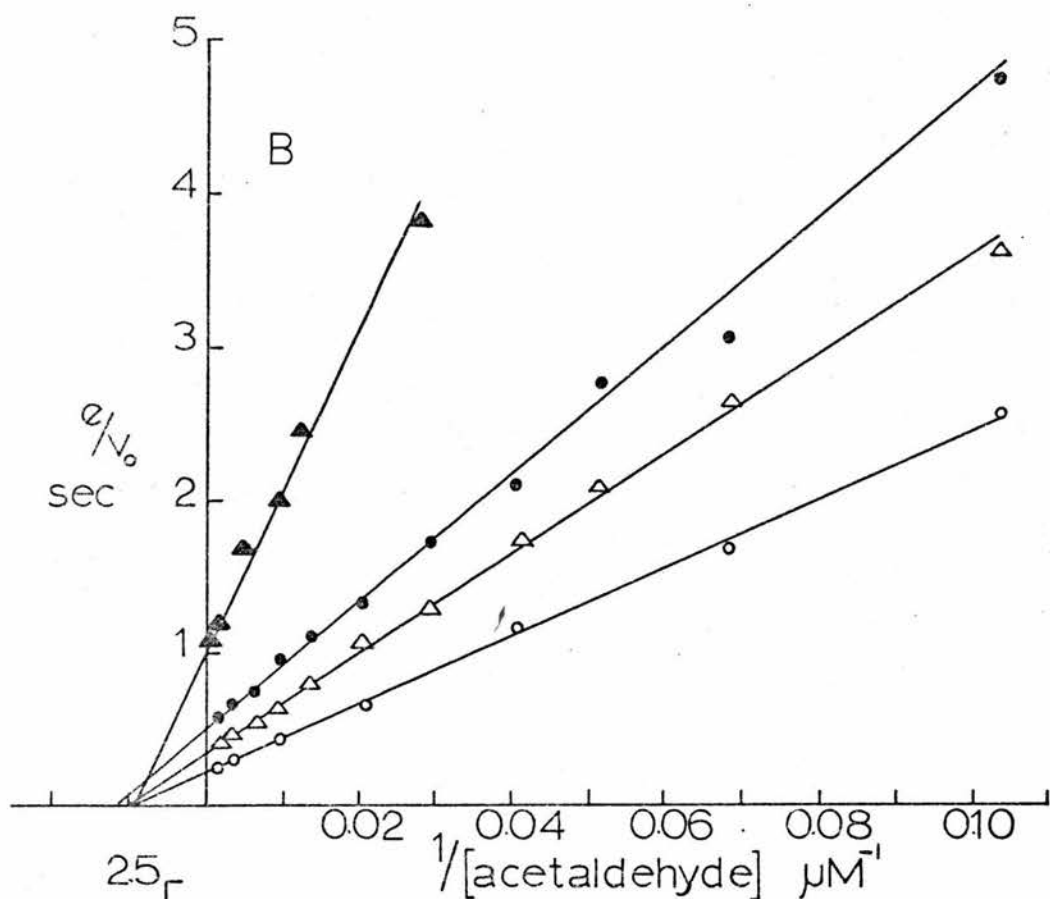


Fig.3-6. Kinetics in the presence of mercaptoethanol at constant DPN^+ concentration ($260 \mu\text{M}$) and varied ethanol concentration (case-2). Buffer, 10mM glycine- NaOH , pH 9.5, plus phosphate to give an ionic strength of 0.1; o, without inhibitor; ●, + mercaptoethanol, $(18 \mu\text{M})$, Δ , $(36 \mu\text{M})$, \blacktriangle , $(72 \mu\text{M})$, ∇ , $(144 \mu\text{M})$.



Kinetics in the presence of Mercaptoethanol at pH 9.5. (Buffer, 10mM glycine NaOH, plus phosphate to give an ionic strength of 0.1).

- A Fig.3-7. Constant acetaldehyde concentration (3.70mM) and varied DPNH concentration (case-3). o, without inhibitor; + mercaptoethanol, \bullet , (180 μ M), Δ , (360 μ M), \blacktriangle , (710 μ M).
- B Fig.3-8. Constant DPNH concentration (13.9 μ M) and varied acetaldehyde concentration (case-4). o, without inhibitor; + mercaptoethanol, Δ , (180 μ M), \bullet , (360 μ M), \blacktriangle , (710 μ M).

The intercepts, however, are clearly increased and inhibition is not therefore abolished by infinite DPN^+ concentration.

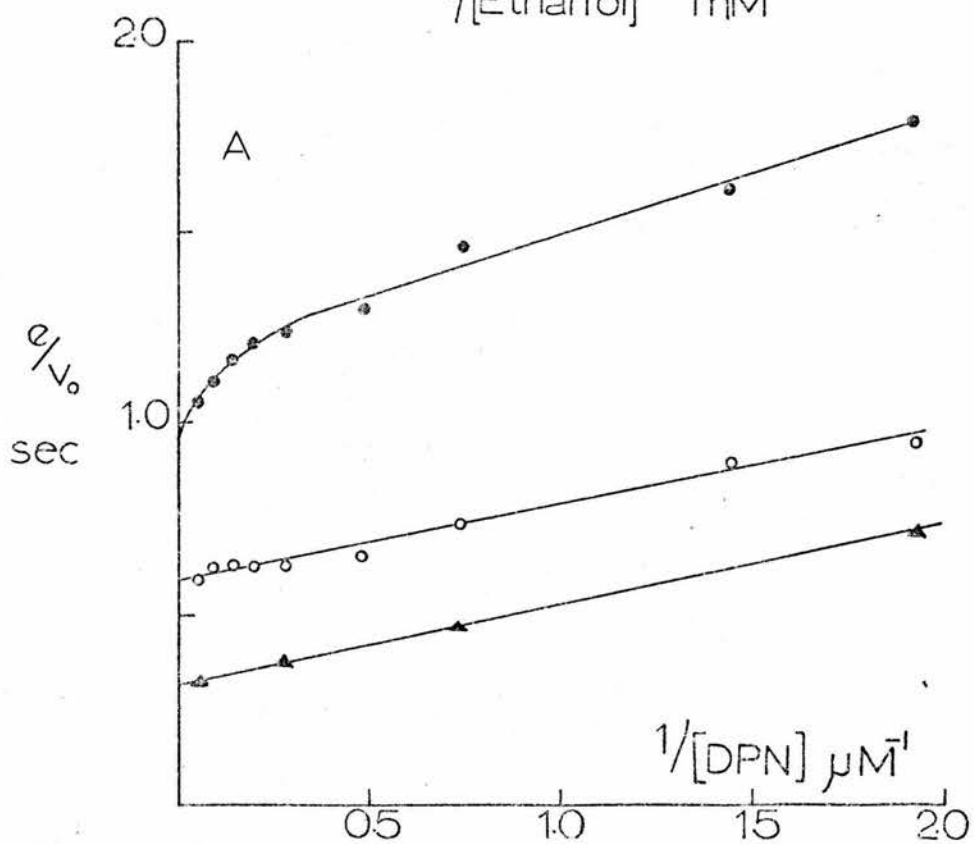
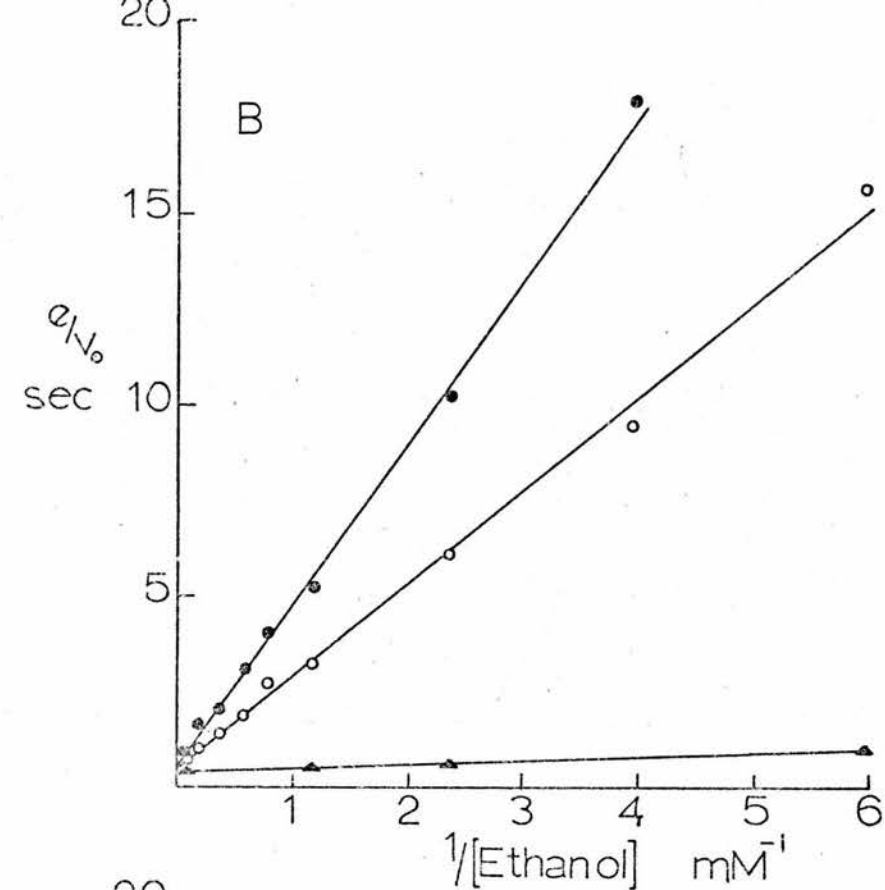
Case-2 (fig. 3-6): ME is not strictly competitive with ethanol, indicating that ER.ME may be formed. Since the lines were concave-down due to ME acting as a substrate, slopes were taken from the tangents at the intercepts. The inhibition constants calculated from the slopes are given in table 3-1.

Case-3 (fig. 3-7): In contrast to pH 7.14 (case-3) the lines are linear. The lines are not parallel and since the acetaldehyde concentration is finite this may be due to the formation of E.ME. The affinity of the thiolate anion for zinc is much greater than that of the undissociated thiol group. The change in the intercept is due to the formation of EO.ME and possibly ER.ME.

Case-4 (fig. 3-8): In contrast to case-4 at pH 7.14 the lines are linear. The increased intercept in the presence of ME is due to formation of EO.ME, as expected from the results of case-2. The increase in the slopes indicates that ME is competitive with acetaldehyde and ER.ME is therefore formed. Values for the inhibition constants calculated from the slopes in order of increasing ME concentration are 380, 430 and 200 μM respectively. The average value for K_I is 340 μM .

Inhibition by ethanethiol

In order to elucidate the problem of the curved



- A Fig.3-9. Kinetics in the presence of ethanethiol at constant ethanol concentration (8.25mM) and varied DPN⁺ concentration (case-1). Buffer, phosphate, pH 7.14, ionic strength 0.1; ▲, without inhibitor; + ethanethiol, ○, (1.7 μM), ●, (2.8 μM).
- B Fig.3-10. Kinetics in the presence of ethanethiol at constant DPN⁺ concentration (260 μM) and varied ethanol concentration (case-2). Phosphate buffer, pH 7.14, ionic strength 0.1; ▲, without inhibitor; + ethanethiol, ○, (1.7 μM), ●, (2.8 μM).

Lineweaver-Burk plots inhibition by ethanethiol was studied. This compound is not a substrate and is a more potent inhibitor than ME. The binding of ethanethiol is thus not as complex and smaller concentrations could be used. Inhibition by ethanethiol is not instantaneous. Indeed, depending on the conditions, EOethanethiol complex is formed over a period of several minutes. Thus at high DPN^+ and ethanol concentrations, when most of the enzyme is in the form ER, the amount of EO is very low and the rate of EOethanethiol formation is slow. When the reaction was started by addition of enzyme the initial velocity was uninhibited, whereas when the reaction was started by the addition of ethanol, after incubation of the enzyme in the reaction cuvette, or by addition of pre-formed EOethanethiol, the initial velocity was nearly zero. In all three cases the final steady state velocity was the same. However, the latter two methods were used in initial velocity measurements when ethanethiol was present. A similar phenomenon has been found for the inhibition of LADH by pyrazole, when EO.pyrazole is formed (Theorell, Yonetani and Sjoberg, 1969), or by n-butyramide, when EO.n-butyramide is formed with a half time of about one minute (Sigman and Winer, 1970). In contrast to ethanethiol, inhibition by ME is instantaneous.

Inhibition by ethanethiol at pH 7.14

Case-1 (fig. 3-9): Inhibition is uncompetitive at low DPN^+ concentrations. At higher ethanethiol concentrations a concave-down curve near the ordinate is evident. At high and low DPN^+ concentrations the extent of inhibition

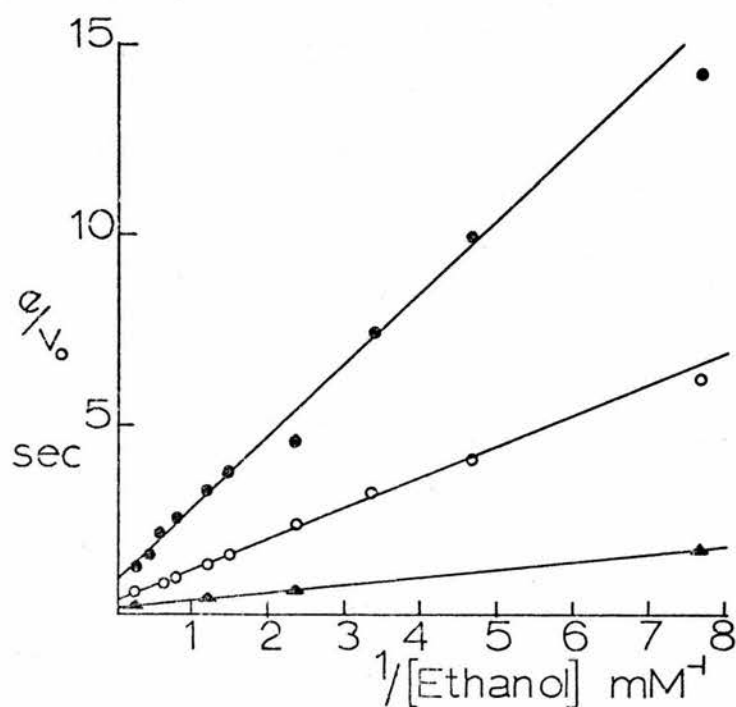
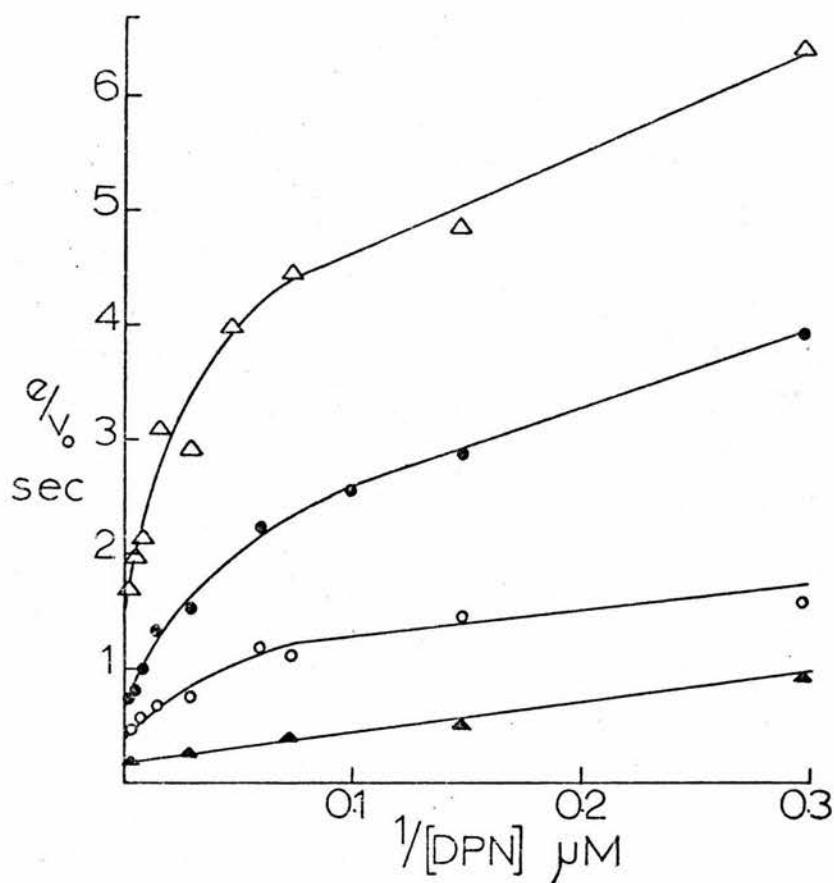


Fig.3-11. Kinetics in the presence of ethanethiol at constant ethanol concentration (8.25mM) and varied DPN^+ concentration (case-1). Buffer, 10mM glycine-NaOH, pH 9.5, plus phosphate to give an ionic strength of 0.1; ▲, without inhibitor; ○, + ethanethiol, (0.1 μM), ●, (0.3 μM), △, (0.6 μM).

Fig.3-12. Kinetics in the presence of ethanethiol at constant DPN^+ concentration (260 μM) and varied ethanol concentration (case-2). Buffer, 10mM glycine-NaOH, pH 9.5, plus phosphate to give an ionic strength of 0.1; ▲, without inhibitor; ○, + ethanethiol, (0.1 μM), ●, (0.3 μM).

is proportional to the ethanethiol concentration. The inhibition constant was also calculated from the intercepts taken as equal to $1/k_2 + \left[1 + (I)/K_I\right] / k_3$ (alc). This expression is derived from the Theorell-Chance mechanism for LADH with inhibition due to formation of an EOI complex included. The rate constants are the same as those given in Chapter 1. The value of k_3 ($0.0122 \text{ sec.}^{-1} \times \mu\text{M}$) determined by Theorell and McKinley-McKee (1961) was used. The intercept with inhibitor, minus the intercept without inhibitor, is equal to $(I)/K_I k_3$ (alc). An average value of $0.06 \mu\text{M}$ was obtained for K_I .

Case-2 (fig. 3-10): Inhibition is strictly competitive and the plots are straight lines. Inhibition constants calculated from the slopes are given in table 3-1. The average value of $0.07 \mu\text{M}$ is 150-fold less than the corresponding constant for ME ($11 \mu\text{M}$). Case-1 and case-2 indicate that inhibition by ethanethiol is due solely to the formation of EO.ethanethiol.

Inhibition by ethanethiol at pH 9.5

The pK_a of ethanethiol has been determined to be 10.0 (Calvin, 1954), 10.61 (Irving et al., 1964) and 10.25 (Lienhard and Jencks, 1964). Thus, assuming the pK to be 10.3 at pH 9.5, about 15% of ethanethiol is thiolate anion.

Case-1 (fig. 3-11): The concave-down curves are very much more pronounced than at pH 7.14. There is also a distinct increase in slope of the asymptote, indicating that a binary E.ethanethiol complex, which excludes DPN^+ binding,

may be formed. The change in intercept is due to the formation of EO.ethanethiol and probably ER.ethanethiol (case-2).

Case-2 (fig. 3-12): The lines are linear. The intercept increases with increasing ethanethiol concentration, probably indicating the formation of ER.ethanethiol as mentioned above. The average value of K_I calculated from the slopes is $0.03 \mu M$.

Spectrophotometric investigation of EO.ethanethiol

Van Eys et al., (1957) reported that formation of a ternary complex of LADH, DPN^+ and ethanethiol generated a difference spectrum with a broad peak of low intensity and an absorption maximum at 315nm. Enzyme was titrated with ethanethiol in the presence of excess DPN^+ , or with DPN^+ in the presence of excess ethanethiol as shown in fig. 3-13 and fig. 3-14. The number of sites titrated in each case is found to be equivalent to the number of co-enzyme binding sites. Millimolar extinction coefficients calculated from the data in figs. 3-13 and 3-14 were found to be 7.30 and 7.50 respectively. An average value of 7.40 was used for subsequent calculations. $K_{EO,I}$ was calculated by the method of Theorell and Yonetani (1963). $K_{EI,0}$ was calculated from an experiment identical to that in fig. 3-14, but with less enzyme. The results are shown in table 3-3. $K_{EO,I}$ was found to be $2.1 \mu M$, which is 30-fold greater than the inhibition constant from case-2 at pH 7.14.

Using the relationship, $K_{EI,0}/K_{E,0} = K_{EO,I}/K_{E,I}$ and

TABLE 3-2

Ethanethiol: The dissociation constants $K_{EO,I}$ and $K_{EI,0}$ were calculated for ethanethiol from spectrophotometric titrations.

(a) Calculations from fig. 3-13.

Ethanethiol	EOI	EO	$K_{EO,I}$
μM	μM	μM	μM
11.5	8.6	7.1	2.4
23.0	15.0	4.4	2.3
34.3	19.1	2.6	2.1
45.5	22.2	1.5	1.6
			Average 2.1

(b) Conditions were identical to those used in fig. 3-14 except that the enzyme concentration was $6.25 \mu N$.

DPN ⁺	EOI	$K_{EI,0}$
μM	μM	μM
4.94	4.50	0.20
5.76	5.21	0.14
6.58	5.80	0.10
7.40	6.00	0.13

Average 0.14

Cuvettes with 1cm light paths were used in these experiments and the millimolar extinction coefficient for $E(DPN^+ \text{ ethanethiol})_2$ was taken as 7.40.

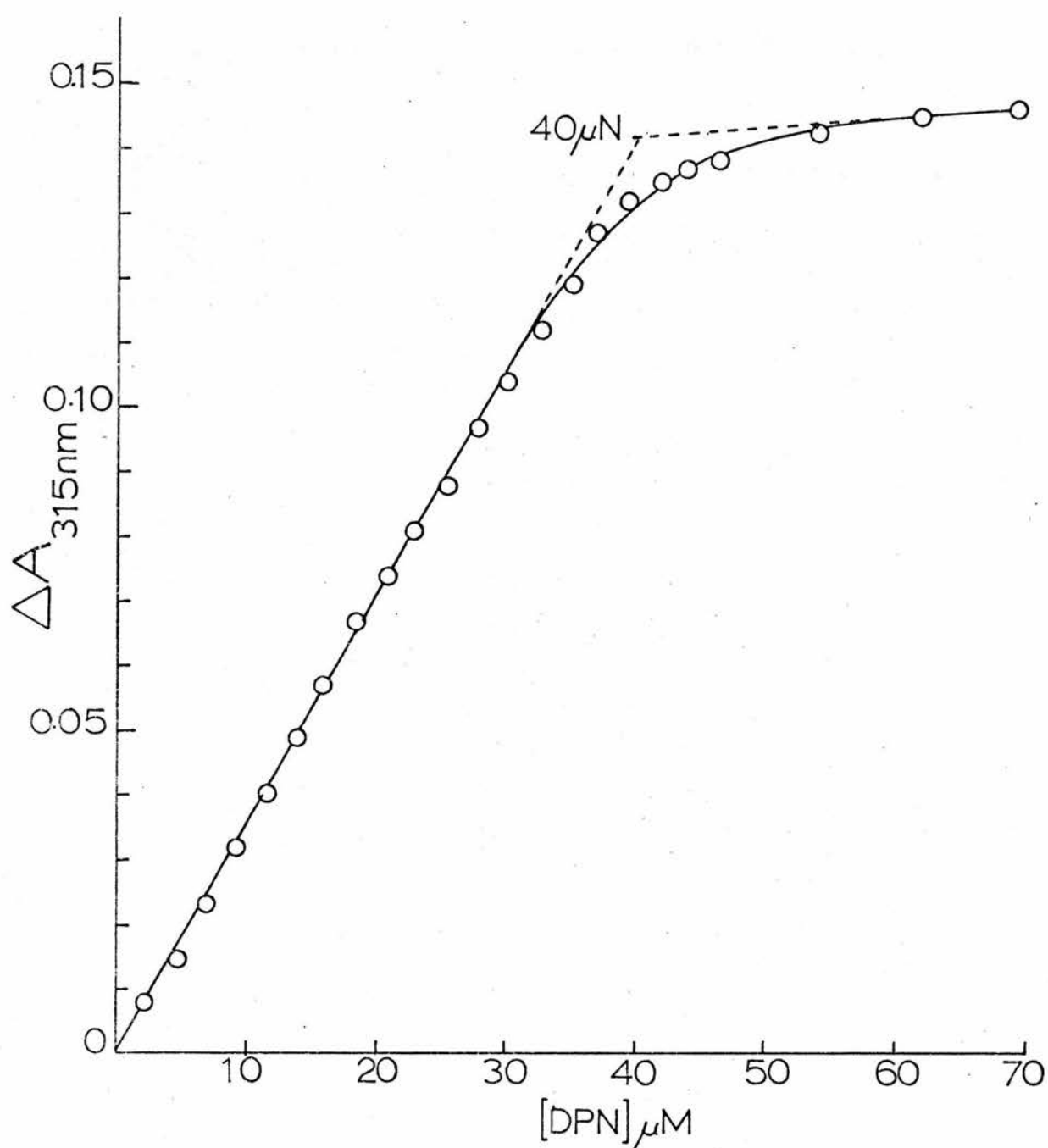


Fig.3-15. Spectrophotometric titration of LADH with DPN^+ in the presence of excess Ethanethiol. Enzyme, $31.5 \mu N$ (determined by fluorimetric titration with $DPNH$ in the presence of isobutyramide); ethanethiol, $15.0mM$; buffer, $33mM$ glycine- $NaOH$, pH 9.5.

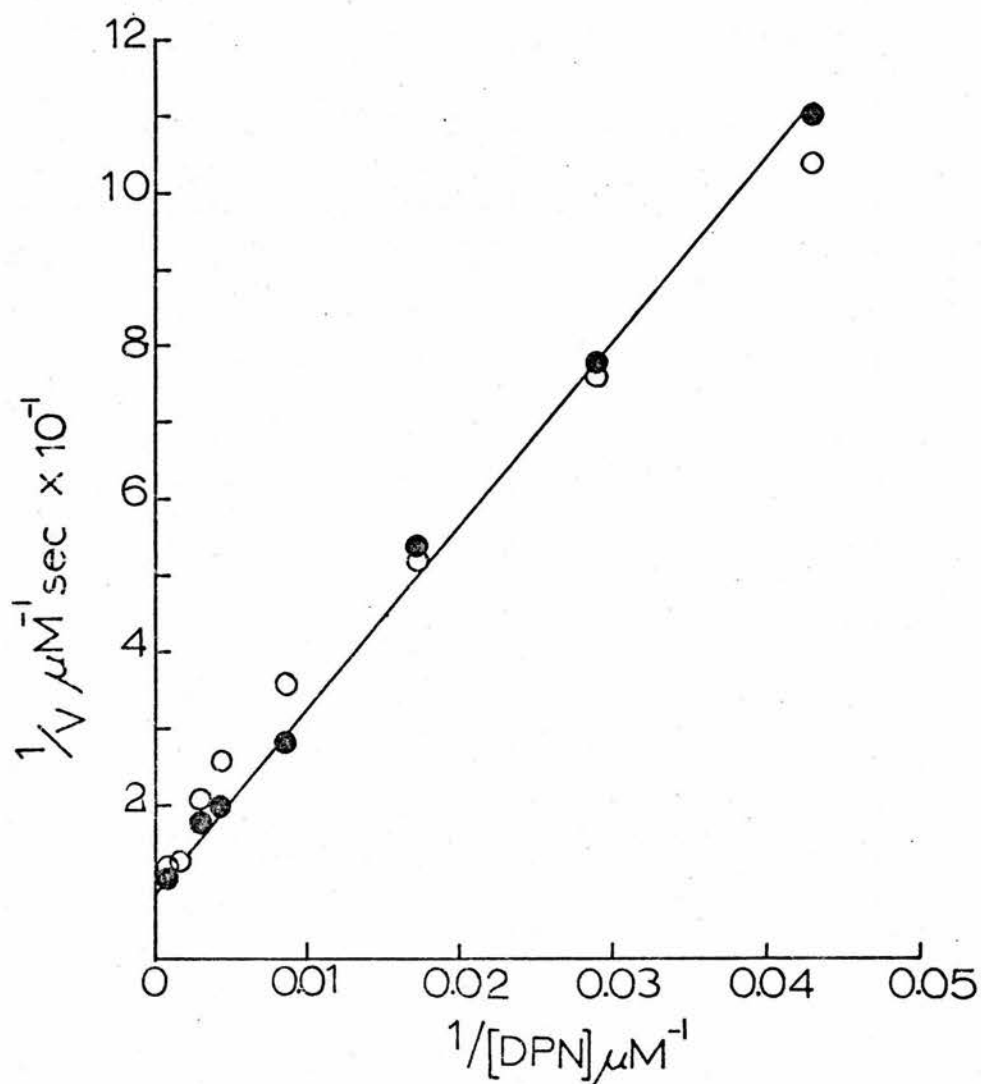


Fig.3-16. Effect of mercaptoethanol on the reduction by DPN^+ by lactate catalysed by Lactate Dehydrogenase (Beef heart). Buffer, 10mM glycine-NaOH, pH 9.5, plus phosphate to give ionic strength of 0.1; lactate, 10.8mM; ●, without mercaptoethanol; ○, + mercaptoethanol (17.85mM).

substituting the values in table 3-3 and $K_{E,0} = 160 \mu\text{M}$ (Theorell and McKinley-McKee, 1961), K_I for ethanethiol was found to be 2.34mM , which is in good agreement with the value of 1.8mM found by Sigman (1967).

To test if a complex between DPN^+ and thiol, which cannot bind to the active centre of LADH, is formed in free solution at pH 9.5, LADH was titrated at pH 9.5 with DPN^+ in the presence of excess ethanethiol (fig. 3-15). The end point of the titration ($40 \mu\text{N}$) was significantly greater than the actual coenzyme binding site concentration ($31.5 \mu\text{N}$). This may indicate that DPN^+ and thiols do indeed combine at high pH to produce a product, which cannot bind to the active centre of LADH. The effect of ME on the reduction at pH 9.5 of DPN^+ by lactate catalysed by lactate dehydrogenase

As shown in fig. 3-16 a high concentration of ME produced no significant inhibition. This is contrary to what would have been expected if DPN^+ was combining with ME to form a catalytically inactive complex. Such a complex would actively inhibit by binding to the enzyme, or produce apparent inhibition by lowering the DPN^+ concentration.

Experiments with other thiols at pH 7.14

Kinetics

Dithiothreitol was strictly competitive with ethanol and the inhibition constant determined from the slopes was $143 \mu\text{M}$. Dithiothreitol showed mixed inhibition in case-1. It would seem therefore that $\text{E} \cdot \text{O}$ dithiothreitol and $\text{E} \cdot \text{dithiothreitol}$ complexes are formed.

Dithiothreitol may be binding at the zinc atom in the active centre competitively with DPN^+ . The value of K_I calculated from the intercepts was $32\mu\text{M}$. At the concentration of dithiothreitol used the plot was linear.

1,2-Ethanedithiol would be expected to have a very high affinity for zinc since a similar compound 2,3-dimercaptopropan-1-ol has a first association constant for zinc, $\log K_1$ of 13.48 (Leussing and Tischer, 1961). 1,2-Ethanedithiol was strictly competitive with ethanol (K_I calculated from the slopes is $0.05\mu\text{M}$) and uncompetitive with DPN^+ . (K_I calculated from the intercepts is 0.09 M). The plot in case-1 was linear.

Coenzyme A ($106\mu\text{M}$) and cysteamine ($1,210\mu\text{M}$) did not inhibit, although the latter might have been expected to chelate the zinc in the enzyme strongly.

Titration

The formation of EOI complexes involving butanethiol, 1,2-ethanedithiol, 1,4-butanedithiol and benzylthiol, was measured spectrophotometrically at 315nm in phosphate buffer, pH 7.14. Titration of LADH by DPN^+ in the presence of excess thiol, or by thiol in the presence of excess DPN^+ established that there is one binding site per subunit for both thiol and DPN^+ in the EOI complex.

Discussion

Concave-down Lineweaver-Burk plots were obtained in the presence of thiol at both pH 7.14 and pH 9.5. The possible side reactions of thiols in the cuvette solutions and the way such reactions could affect the Lineweaver-Burk plots are considered.

Thiols are susceptible to oxidation and this may be significant when very low concentrations of thiols are used as in the experiments with ethanethiol and ME, at pH 9.5 in particular. However, several observations indicate that under the conditions of these experiments although oxidation was occurring it was too slow to be a serious problem.

- (a) Titrations of LADH in the presence of excess DPN^+ with a variety of thiols at pH 7.14 gave the correct coenzyme binding site concentration. As each titration took about 30 minutes to perform this indicates that the rate of destruction of free thiol by oxidation at pH 7.14 was negligible.
- (b) At pH 9.5 a reduction of inhibition was observed, dependent on the time the cuvette solution containing thiol was incubated before the initial velocity determination. However, in initial experiments cuvette solutions were prepared in duplicate, simultaneously and the second incubated while the first was utilized. On average the second cuvette was used 6 minutes after the first. In the case of ethanethiol the initial velocities obtained with the two solutions agreed within normal experimental error. Mercaptoethanol appeared to be oxidised more rapidly and a constant increase in initial velocity of about 10% was observed with the second cuvette compared to the first. It was therefore assumed that if thiol was added to the cuvette solution just prior

to the enzyme oxidation of thiol could be disregarded as a serious side reaction.

- (c) The initial velocity progress curves obtained at pH 9.5 in the presence of mercaptoethanol were linear for a minute or longer. If thiol was being removed by oxidation a time dependent increase in velocity would have been observed except in the unlikely coincidence of the product of oxidation of the thiol having exactly the same potency as an inhibitor.
- (d) At pH 9.5 the K_I values calculated from the slopes in case-2 for ME and ethanethiol do not change much with varied thiol concentration (table 3-1). If oxidation of thiol was serious the value of K_I would decrease appreciably as the thiol concentration was increased.

It is concluded that the curved Lineweaver-Burk plots are not caused by oxidation of the thiols in the cuvette solution. This is supported by the observation that a concave-down plot was obtained in case-1 at pH 7.14 in the presence of ethanethiol (fig. 3-9), whereas it appears (see (a) above) that oxidation is not significant. Moreover, in fig. 3-9 there is no significant curvature in the presence of the lower ethanethiol concentration although oxidation might be expected to become more serious as the thiol concentration is reduced.

It has been shown that thiols can react with DPNH in an acid catalysed reaction by adding across the 5,6 double bond of the nicotinamide ring (Wallenfels and

Schüly, 1957). However, this reaction abolishes DPNH fluorescence, whereas it was found that at pH 7.14 a plot of DPNH concentration against fluorescence was unaltered by the presence of $710 \mu\text{M}$ ME.

ME and ethanethiol react with acetaldehyde to form hemithioacetals (Lienhard and Jencks, 1966). However, at the highest concentrations of thiol ($710 \mu\text{M}$ ME) and acetaldehyde ($3,700 \mu\text{M}$) present in the kinetic experiments the amount of hemithioacetal produced would be expected to be very small.

It is known that thiols, regardless of structure, form complexes with DPN^+ (Van Eys and Kaplan, 1957; Wallenfels and Schüly, 1957). For ethanethiol the equilibrium constant,

$$K = \frac{(\text{DPN}^+ \text{ ethanethiol})(\text{H}^+)}{(\text{ethanethiol})(\text{DPN}^+)}$$

$$= 2.4 \times 10^{-11}, \quad \text{has been determined}$$

by Van Eys and Kaplan (1957). Thus at pH 9.5 at the maximum concentrations of DPN^+ (approx. $1000 \mu\text{M}$) and ethanethiol ($0.6 \mu\text{M}$) which were employed the concentration of DPN^+ -thiol complex was of the order of $10^{-11} \mu\text{M}$ which is two orders of magnitude lower than the concentration of enzyme in the solution. If it is assumed that ME behaves similarly, at the maximum concentration of DPN^+ (approx. $1000 \mu\text{M}$) and ME ($90 \mu\text{M}$) the concentration of DPN^+ -thiol complex was of the order of $10^{-9} \mu\text{M}$. Therefore the concentrations of DPN^+ -thiol complex formed under the conditions of these experiments was too small to have an inhibitory effect, either by binding to the enzyme

competitively with DPN^+ , or by sequestering DPN^+ in a catalytically inactive form. This latter conclusion is supported by the failure of a high ME concentration to inhibit lactate dehydrogenase when the DPN^+ concentration was varied.

The possibility that the curved Lineweaver-Burk plots are caused by an effect produced in the enzyme by the binding of ethanethiol or ME at the active centre must be taken into consideration. Although the binding of thiol to form an EOI complex at one active centre of the dimeric enzyme molecule completely inhibits that site changes may be transmitted through the molecule which alter the properties of the distant uninhibited active centre. This consideration is discussed in Chapter 9.

The K_I values presented in table 3-1 show little variation with pH. The formation of an EOI complex in which the thiol is bound to the active centre zinc, or forms a C-4 adduct or a charge transfer complex with enzyme bound DPN^+ , should be strongly favoured by increased pH. Olander and Kaiser (1971) have shown that thiophenols bind to the zinc atom in carbonic anhydrase 1000-fold more tightly at pH 10 than at pH 7. The pH independence of K_I may, however, be more apparent than real due to the effect of varying DPN^+ concentration on the inhibition by ME and ethanethiol. One can obtain K_I values from the intercepts with the ordinate of the asymptotes to the curves in fig. 3-5 and fig. 3-11 using the method applied to fig. 3-9. With k_3 taken as $0.00889 \text{ sec.}^{-1} \times \mu\text{M}$ (Theorell and McKinley-McKee, 1961 a), K_I for ME has the value $0.5 \mu\text{M}$

for both curves in fig. 3-5 while K_I for ethanethiol has the value $0.002\mu\text{M}$ for all three curves in fig. 3-11. At pH 7.14 K_I values of $5\mu\text{M}$ for ME and $0.06\mu\text{M}$ for ethanethiol are obtained from fig. 3-1 and fig. 3-9 respectively. Thus the K_I for ME decreases 10-fold and the K_I for ethanethiol decreases 30-fold when the pH is increased from 7.14 to 9.5. The significance of these K_I values is obscured by the fact that ME is a substrate, which results in an apparently smaller extent of inhibition at V_{max} and EO.thiol complexes are formed at pH 9.5 which leads to an additional increase in the intercept. However, although these K_I values do not represent $K_{\text{EO,thiol}}$ they do indicate that $K_{\text{EO,thiol}}$ is pH dependent.

The results of these kinetic experiments indicate that an EO.thiol complex is formed, but they cannot decide whether the thiol is binding to the active centre zinc or forming a complex with enzyme bound DPN^+ . The similar values for K_I obtained with ethanethiol and 1,2 ethanedithiol indicate that the active centre zinc may not be involved in binding thiols since 1,2 ethanedithiol is bound by free zinc ions much more tightly than ethanethiol. Alternatively, however, an EO.thiol complex in which zinc is in a tetrahedral configuration with three zinc-protein bonds and one valence free to bind thiol could result in 1,2 ethanethiol bound to zinc by only one of its thiol groups. In this case 1,2 ethanedithiol and ethanethiol would be expected to bind with equivalent strength. It is entirely feasible however, that the binding site of thiols in the EO.thiol complex is the C-4 position of DPN^+ . Everse et al.,

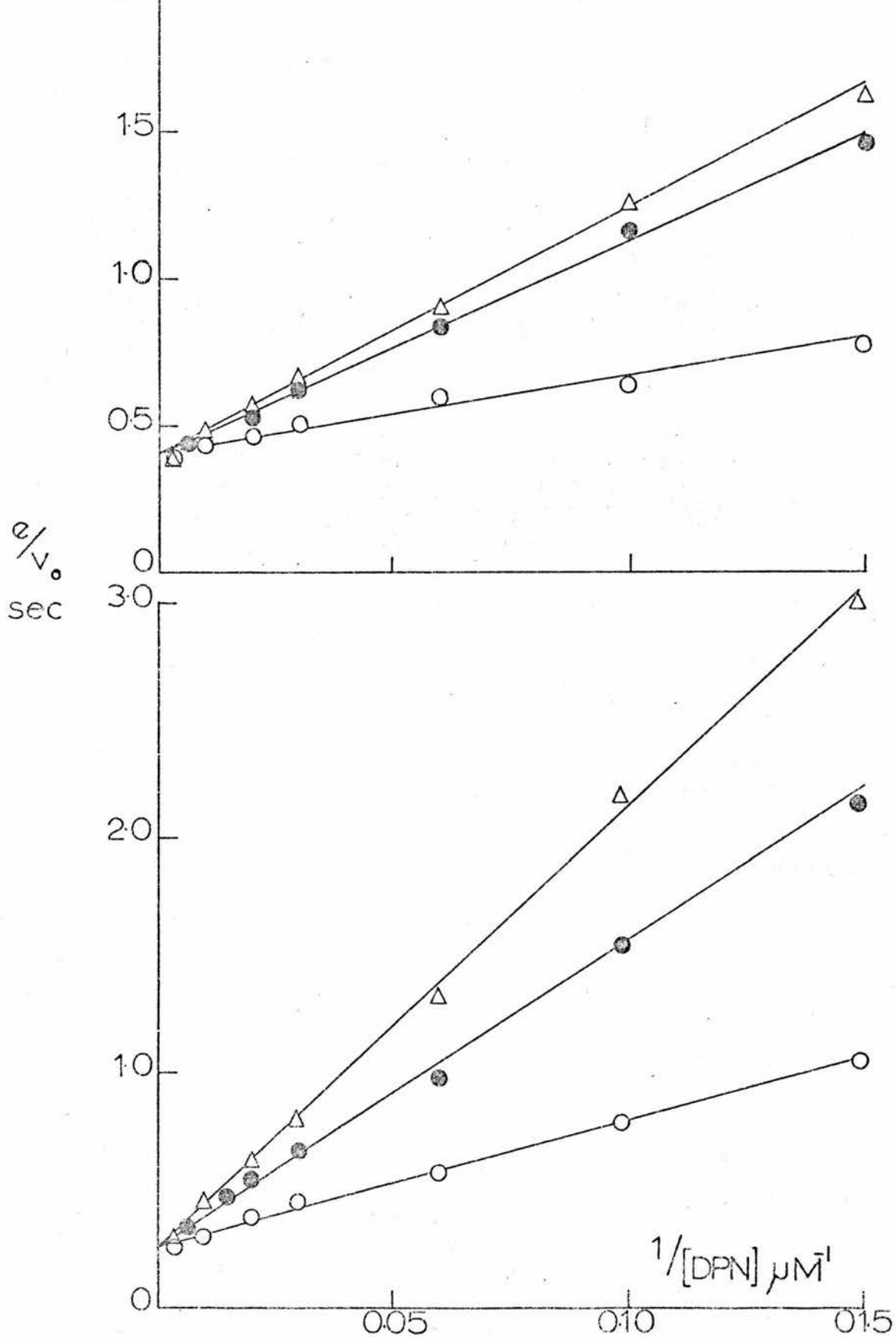
(1971) have isolated C-4 addition products of DPN^+ with acetaldehyde and butyraldehyde that bind to the enzyme with a stoichiometry of two molecules of DPN^+ adduct per LADH molecule. Thus although adducts of DPN^+ and thiol are formed in free solution to a negligible extent it may be that such adducts are greatly stabilised by interaction with the active centre of LADH. Moreover, although the thiols may be bound covalently to DPN^+ in the EO thiol complex, this does not rule out binding of alcohols to the zinc. Thiols were shown to be strictly competitive with ethanol and the strength of their binding to increase with increased hydrophobicity of the thiol. It is probable that the hydrophobic tails of ethanol and the thiols bind competitively at the same hydrophobic site in the active centre. The thiol and hydroxyl groups need not in this event bind at the same site. Since sulphur is more polarizable and nucleophilic than oxygen the thiols may bind preferentially and covalently to C-4 of the nicotinamide ring of DPN^+ whereas the hydroxyl groups of alcohols may bind preferentially to the active centre zinc.

In the ER.thiol complex, shown to be formed at pH 9.5 but not detected in experiments at pH 7.14, the active centre zinc may be implicated as a binding site for the thiol with more confidence. Covalent addition of the thiol to the enzyme bound DPNH is unlikely as this is an acid catalysed reaction and would thus occur more readily at pH 7.14. The undissociated thiol rather than the thiolate anion is perhaps the predominant species bound since anions are generally competitive with ethanol but not with

acetaldehyde. For example, decanoate forms an EOI but not an ERI complex (Winer and Theorell, 1960), and chloride is competitive with ethanol but uncompetitive with acetaldehyde (Theorell et al., 1955). The active centre zinc is considered to have a bound water molecule with a pK_a of about 8.6 in the free enzyme (Theorell and McKinley-McKee, 1961 b; Taniguchi, Theorell and Åkeson, 1967). The binding of DPNH to LADH decreases markedly when the pH is increased above 9 (Theorell and Winer, 1959) and it may be that although $ER.H_2O$ is much more stable than $ER.thiol$, $ER.thiol$ is more stable than $EROH^{(-)}$. This could explain why $ER.thiol$ becomes significant as the pH is increased. However, an $ER.thiolate$ complex is by no means ruled out and would not have been detected at pH 7.14 due to the extremely low concentrations of ionized mercaptoethanol or ethanethiol.

CHAPTER 4.

The AMP Binding Site in Liver Alcohol Dehydrogenase



Kinetics in the presence of AMP and adenosine at constant ethanol concentration (8.25mM) and varied DPN^+ concentration.

Fig.4-1. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; o, without inhibitor; ●, + AMP (132 μM); Δ, + adenosine (12.81mM).

Fig.4-2. Buffer, 10.0mM glycine-NaOH plus phosphate, pH 10.0, ionic strength 0.1; o, without inhibitor; ●, + AMP (660 μM); Δ, + adenosine (14.60mM).

Results and Discussion

In order to assess the importance of the phosphate group of AMP in binding the inhibitor to the active centre of LADH, the pH dependence of AMP and adenosine binding was investigated. The kinetics of the reduction of DPN^+ by ethanol was used to determine inhibition constants for AMP and adenosine at pH 7.14 and pH 10.0 (table 4-1). Fig. 4-1 and fig. 4-2 show that AMP and adenosine are strictly competitive with DPN^+ at both pH values. Adenosine binds much more weakly than AMP and its binding is pH independent, whereas the inhibition constant for AMP increases by 6.7-fold from pH 7.14 to pH 10. This indicates that the phosphate group has a strong influence on AMP binding and it is possible that a positively charged amino acid residue on the enzyme is involved. This amino acid residue is not the only ionizable group involved in AMP binding since imidazole strengthens the binding of AMP to LADH at pH 10 but does not abolish the pH dependence (Reynolds et al., 1970). This was interpreted as indicating that the active centre zinc helps to bind AMP since imidazole has been shown to bind to the active centre zinc (Sigman, 1967), which is considered to have a bound water molecule with a pK_a of about 8.6 (Theorell and McKinley-McKee, 1961 b, Theorell et al., 1967). The postulated positively charged amino acid residue may be a lysine which normally have pK_a s in the range 9.5 - 10.5 in proteins. It has previously been postulated that a

TABLE 4-1

Kinetic determination of inhibition constants for AMP and adenosine at pH 7.14 and pH 10.0.

At pH 7.14 evaluated from the slopes in fig. 4-1.

At pH 10.0 evaluated from the slopes in fig. 4-2.

Inhibitor	Inhibition constant (μM)	
	pH 7.14	pH 10.0
AMP	69	460
Adenosine	6,200	5,800

lysine residue may be involved in binding ADP-ribose to LADH (Fisher et al., 1967).

CHAPTER 5.

Chemical Modification of the Lysines of Liver
Alcohol Dehydrogenase by Pyridoxal-5'-Phosphate

Introduction

It has been previously postulated (Chapter 4) that a positively charged amino acid residue, perhaps a lysine, at the binding site of the coenzyme, serves to attract the phosphate group of the AMP moiety of the coenzyme. The selective chemical modification of lysine groups in LADH is one approach which could be used to investigate this theory. Also, it has been suggested that lysine residues are involved in binding ADP-ribose to LADH (Fisher et al., 1967) and that a lysine is situated in the active centre close to where the ring nitrogen of the coenzyme nicotinamide moiety is bound (Kosower, 1962).

PLP has been found to be a highly specific reagent for the chemical modification of lysine residues in a variety of enzymes. PLP is bound by formation of a readily reversible Schiff's base with the ϵ -amino group of lysines and can be permanently incorporated into the protein by reduction of the Schiff's base with sodium borohydride. The following enzymes have been inactivated by incorporation of PLP groups: glutamate dehydrogenase (Anderson et al., 1966), rabbit muscle aldolase (Shapiro et al., 1968), 6-phosphogluconate dehydrogenase (Rippa et al., 1969), glyceraldehyde-3-phosphate dehydrogenase (Ronchi et al., 1969), rabbit muscle pyruvate kinase (Johnson and Deal, 1969), adenylic acid deaminase (Kaldor and Weinbach, 1966) and hexokinase (Grillo, 1968). PLP has also been shown to desensitize fructose 1,6 diphosphatase to allosteric inhibition by AMP (Marcus and Hubert, 1968), to lower the oxygen affinity of haemoglobin (Benesch et al., 1969) and

used to identify at least three classes of lysine which bind PLP to bovine plasma albumin (Dempsey and Christensen, 1962).

It was hoped that the phosphate group might confer some specificity on the reaction of PLP with lysine groups in LADH by directing the molecule into the AMP binding site.

Despite the high specificity exhibited in the above studies it is not safe to assume that PLP will react solely with ϵ -amino groups of lysine in a protein since it has been shown that pyridoxal reacts with cysteine to give a thiazolidine derivative and with histidine to give a condensation product which resisted hydrogenation (Heyl et al., 1948) and PLP reacts with various amino thiols to give thiazolidine derivatives (Buell and Hansen, 1960; McKay, 1963).

Methods

Reversible Inactivations

Inactivations were performed at 23.5°C in phosphate buffer, pH 8.0, with the ionic strength maintained at 0.1 by varying the concentration of phosphate. Inactivations were followed by measuring the initial velocity of DPNH formation produced by adding aliquots of the inactivation solution to 3 ml. of assay solution which consisted of 8.25mM ethanol, 350 μ M DPN⁺ and 62mM bicarbonate buffer, pH 10, at 23.5°C. Initial velocities were measured spectrophotometrically by following the increase in absorbance at 340nm on a Gilford 2000 recording spectrophotometer. The 100% value was obtained by measuring the activity of a control solution which

did not contain PLP but was otherwise indential to the appropriate inactivation solution.

Reduction of the reversible PLP-LADH complex

LADH was incubated with the required concentration of PLP plus any additional substances in 0.1M N-ethylmorpholine, HCl, pH 8.0, 23.5°C for fifteen minutes. In order to prevent frothing 2 μ l of octyl alcohol were added. Reduction was achieved by the addition of a few grains of sodium borohydride, stirred in rapidly on the end of a glass stirrer. The small glass phials in which the inactivations were performed were tapped frequently after reduction to help prevent formation of froth. The solution was then transferred to a dialysis sac and dialysed for 48 hours at 4°C against two litres of phosphate buffer, pH 7.4, ionic strength 0.1, with buffer changes after 12 hours and 24 hours.

The number of PLP groups incorporated into the enzyme was determined by measuring the absorbance at 325nm, using a molar extinction coefficient of 10,700 at 325nm (Fischer et al., 1963). This assumes that the pyridoxylphosphate enzyme should have a similar absorbance to ϵ -pyridoxylphosphate lysine. The concentration of reduced PLP-LADH was determined after dialysis by alkaline hydrolysis and ninhydrin analysis (Freucher and Crestfield, 1965; Moore, 1968). An experimentally determined colour value of 6.3 μ M Moles of leucine per mg. of enzyme was used. The enzyme concentration was also estimated by measuring the volume of the dialysate with carlsberg pipettes, thus obtaining the amount of dilution and applying

this correction to the initial concentration.

The activity of the modified enzyme was determined using the Dalziel assay. The activity was then quoted as a percentage of the activity of the unmodified enzyme.

Amino Acid Analysis

Protein samples were hydrolysed in 6N HCl at 105°C for 24 hours after freezing and sealing under vacuum to remove oxygen. Automatic amino acid analysis was carried out by the accelerated method of Spackman, Moore and Stein (1958) on 1.2 mg. protein in the Locarte amino acid analyser.

Polacrylamide Gel Electrophoresis

15% Acrylamide gels prepared according to the receipe of Cruft (1962) were used. Electrophoresis was carried out in the Shandon apparatus. At pH 7, 0.01M phosphate buffer was used for making gels and filling resevoirs outside electrode compartments, whereas, 0.1M phosphate was used inside electrode compartments. At pH 8.7, 0.03M borate buffer was used for making gels and filling resevoirs outside electrode compartments, whereas 0.3M borate buffer was used inside electrode compartments. Electrophoresis was carried out using a constant current maintained at 5mA per tube. The voltage required was about 100 volts at pH 7, and 50 volts at pH 8.7.

The gels were stained for alcohol dehydrogenase activity by incubating in a solution of the following constitution:-
30 ml. of 0.1M glycine NaOH; pH 10.0; 150 μ l ethanol;
6 mg. DPN⁺; 1 mg. N-methylphenazonium methosulphate and
10 mg. nitroblue tetrazolium.

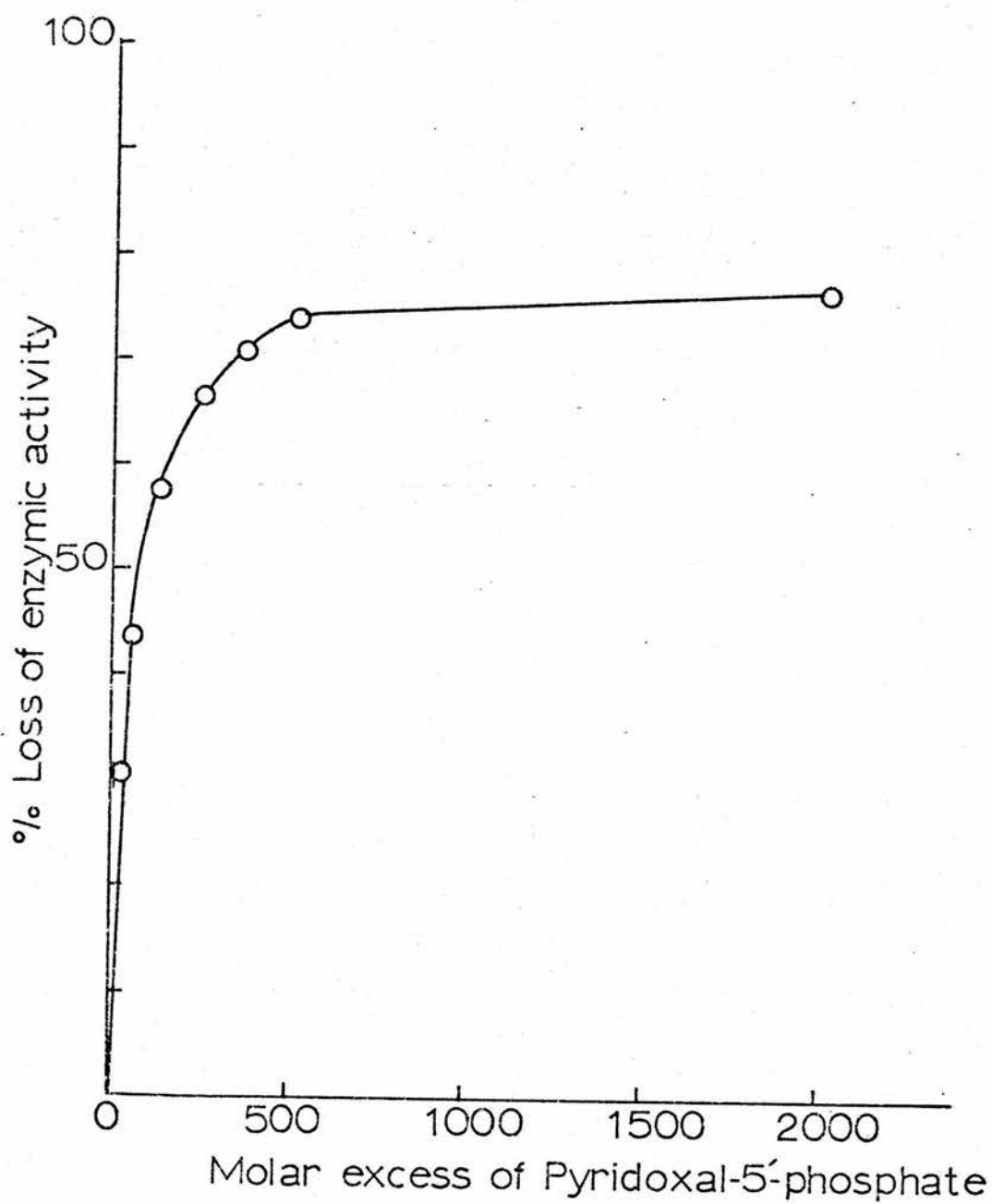


Fig.5-1. The effect of PLP concentration on the extent of the reversible inactivation of LADH ($2\mu\text{N}$) at pH 8.0.

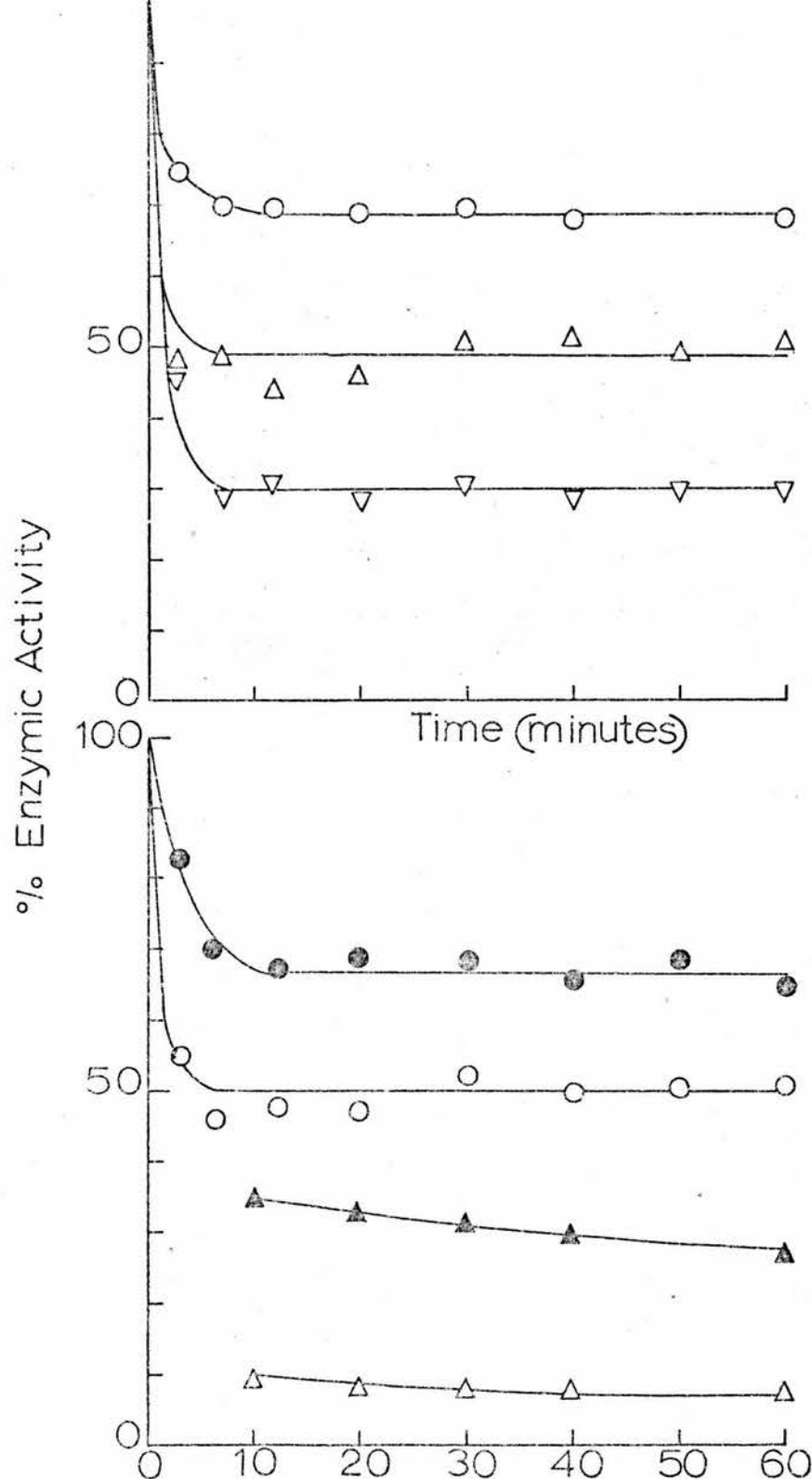


Fig.5-2. The reversible inactivation of LADH by PLP at different pH values. Enzyme, $1.7\mu\text{N}$; phosphate buffer, ionic strength 0.1; PLP, 3.77mM ; 23.5°C ; \circ , pH 6.29; Δ , pH 7.26; ∇ , pH 7.98.

Fig.5-3. The effect of ionic strength on the reversible inactivation of LADH by PLP and benzaldehyde. Enzyme, $1.7\mu\text{N}$; 23.5°C ; \bullet , PLP (7.45mM), phosphate buffer, pH 7.1, ionic strength 1.0; \circ , PLP (7.45mM), phosphate buffer, pH 7.1, ionic strength 0.1; \blacktriangle , benzaldehyde (9.72mM), phosphate buffer, pH 7.1, ionic strength 1.0; \triangle , benzaldehyde (9.72mM), phosphate buffer, pH 7.1, ionic strength 0.1.

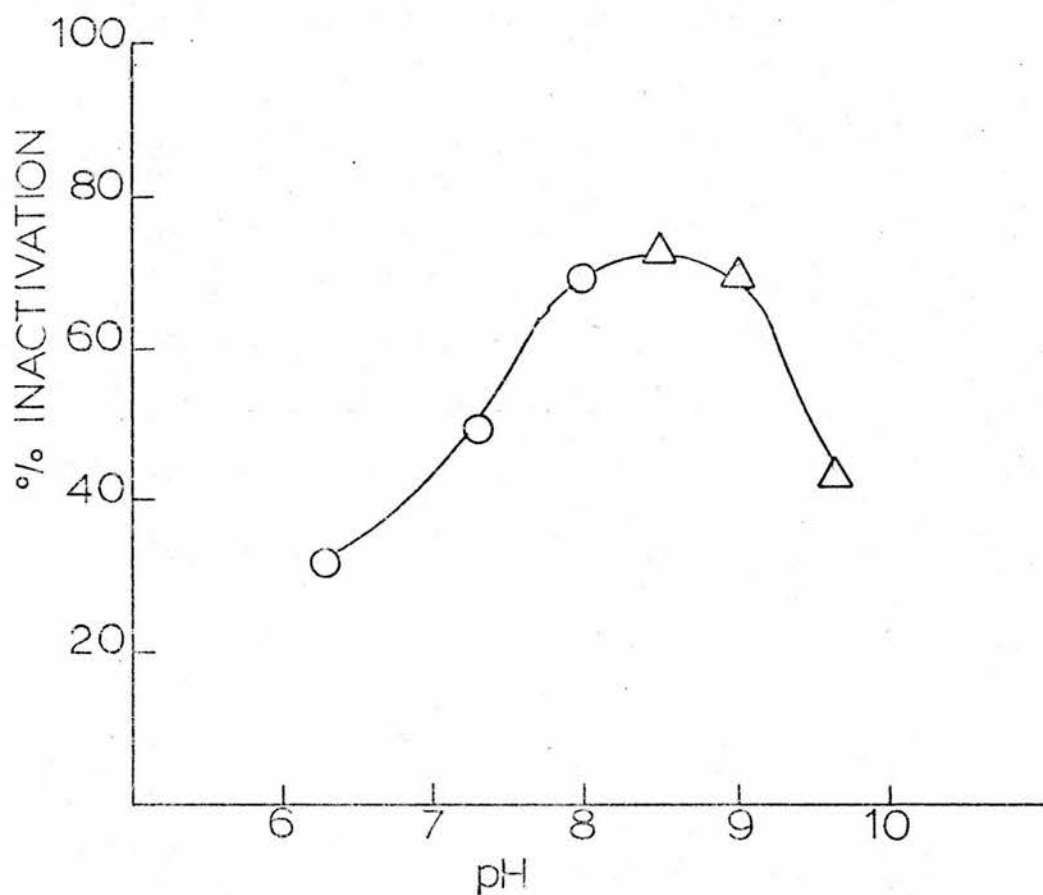


Fig.5-4. The effect of pH on the reversible inactivation of LADH by PLP. Enzyme, $1.7\mu\text{N}$, was incubated with 3.77mM PLP at 23.5°C for 20 minutes. o, phosphate buffer; Δ , pyrophosphate buffer.

The gels were stained for protein with Naphthalene Black 10B in water-methanol-acetic acid (5.4.1) for two hours and then washing with water-methanol-acetic acid (5.4.1). The electrophoretic experiments were performed with the invaluable help of Mr. Graham Pettigrew.

Results

Reversible Inactivation

Incubation of LADH with various concentrations of PLP at pH 8.0 in phosphate buffer led to time-dependent loss of activity which reached final values in about 10 minutes. Typical curves at different pH values are shown in fig. 5-2 and fig. 5-3. Prolonged incubation resulted in no further activity change, so it can be assumed that these represent equilibrium values. A plot of the equilibrium activity values against the molar excess of PLP over LADH is shown in fig. 5-1. A saturation curve was obtained with a maximum activity loss approaching 80%. The inactivation of LADH by PLP was shown by Cheng (1970) to be fully reversible since dialysis of the PLP-LADH complex restored almost full activity. Also, during inactivation experiments, it was observed that the PLP-LADH complex was dissociating on dilution into the assay cuvette since a steady increase in the rate of DPN^+ reduction was observed. The extent of PLP-LADH complex formation was found to be pH dependent with maximum formation between pH 8 and pH 9 (fig. 5-4). pH 8.0 was chosen for most of the subsequent studies.

The effect of phosphate

These experiments are shown in fig. 5-3. When the

ionic strength of an inactivation solution containing 7.45mM PLP at pH 7.1 was increased from 0.1 to 1.0 by varying the phosphate concentration the loss of activity was decreased from 50% to 35%. However, the extent of inactivation of the enzyme by 9.72mM benzaldehyde at pH 7.1 was also decreased by raising the ionic strength. When the ionic strength was increased from 0.1 to 1.0 by varying the phosphate concentration the extent of inactivation, after incubation for one hour, decreased from 91% to 72%. It would seem that phosphate binding to the enzyme protects against inactivation by PLP and benzaldehyde, although benzaldehyde being a neutral molecule should not have its binding at a lysine residue weakened by increased ionic strength. If the phosphate of PLP is being attracted to a positive centre at the active site, increased ionic strength would be expected to weaken this attraction.

Reduction of the reversible PLP-LADH complex with sodium borohydride

Cheng (1970) showed that the reduced pyridoxal-5'-phosphate alcohol dehydrogenase complex had an absorption maximum at 325nm as expected for the formation of reduced Schiff bases between PLP and ϵ -NH₂-groups of lysine residues (Fischer et al., 1959). Direct identification of ϵ -PLP-lysine residues in hydrolysates of the reduced PLP-LADH complex was made. ϵ -PLP lysine was prepared by treating α -N-carbobenzoxyl lysine with a 100-fold molar excess of PLP in phosphate buffer, pH 8.0 at 23.5°C for 30 minutes and then reducing by addition of a few grains of sodium borohydride. The carbobenzoxyl group blocking the α -amino



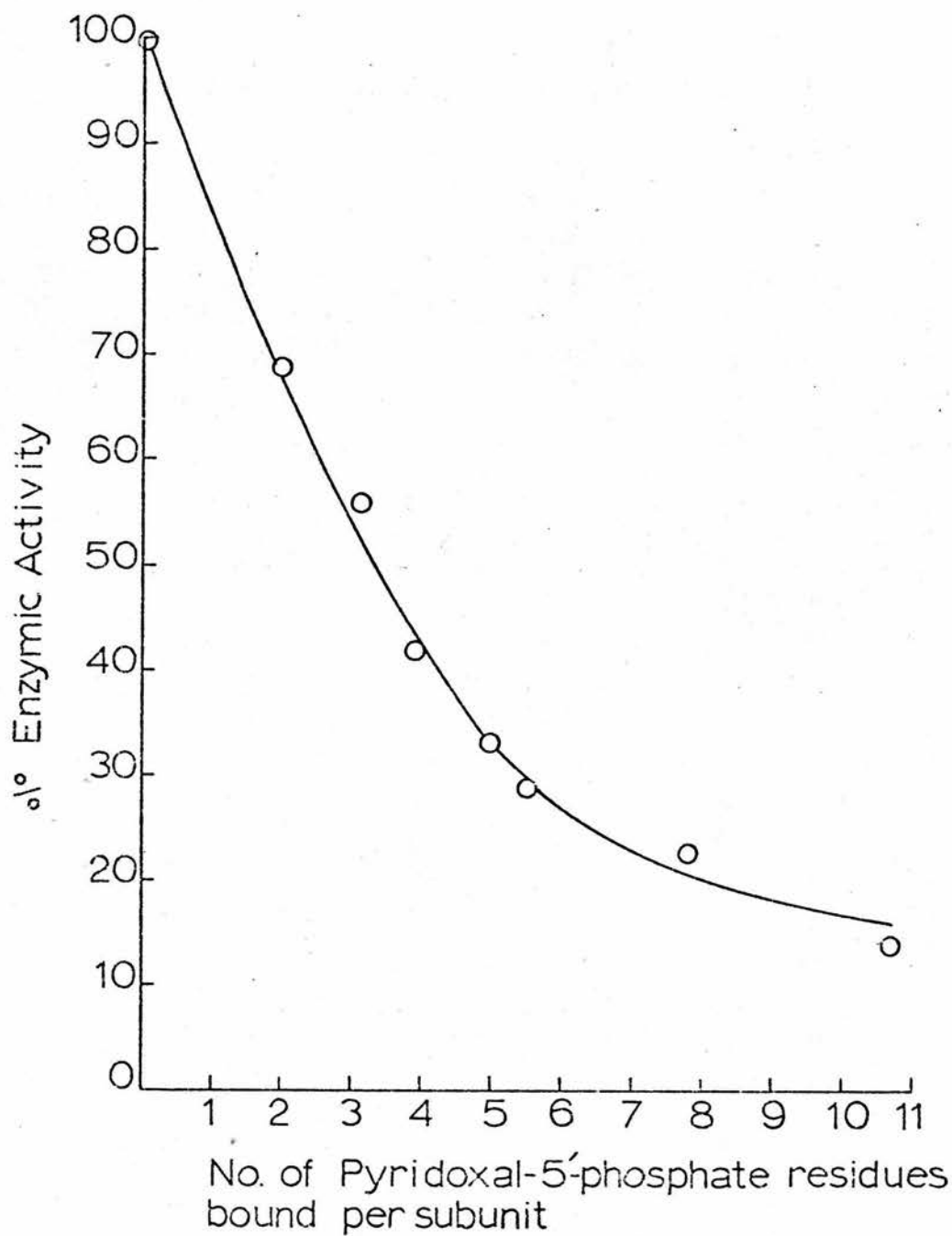


Fig. 5-5. The relation between the number of PLP residues covalently incorporated into LADH and the residual enzyme activity.

group is removed by the reduction. The product was then treated in vacuo with 6N HCl for 24 hours at 105°C and desalted by chromatography on Dowex 50, evaporated to dryness and dissolved in a small amount of distilled water. Analysis in the amino acid analyser revealed a peak at a slightly more alkaline position than lysine. Amino acid analysis of hydrolysates of reduced PLP-LADH revealed a peak corresponding to the model compound which is absent from hydrolysates of native enzyme. No other unexplained peaks appeared in the amino analysis of the reduced complex.

The number of PLP groups incorporated into LADH was determined spectrophotometrically at different PLP concentrations and plotted against the corresponding residual activity (fig. 5-5). A maximum inactivation of about 80% was achieved when six to seven PLP groups per subunit had been incorporated. Increasing the number incorporated to ten to eleven groups required very large molar excesses of PLP and only further reduced the activity by a small amount. 50% inactivation was reached when about 3.5 of the 30 lysine residues per subunit of the enzyme had been modified with PLP. Thus some lysine residues bind PLP more strongly than others.

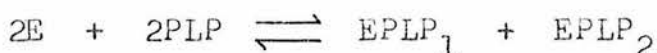
The effect of inhibitors on the reversible inactivation

Various inhibitors were found to decrease the extent of inactivation at equilibrium.

The reversibility of the formation of the PLP-LADH complex and the fact that the extent of inactivation is not proportional to PLP concentration but shows a saturation

effect suggested an analogy with Michaelis-Menten-type kinetics. Indeed, a plot of the reciprocal of molar excess of PLP against the reciprocal of % loss of enzyme activity proved to be a straight line. This also suggests that the binding of one PLP residue per active site is responsible for inactivation since if say two PLP residues per active site were responsible this would require a plot of the reciprocal of $(\text{PLP})^2$ against the reciprocal of loss of enzyme activity to be a straight line, which is not the case.

The following scheme is a possible explanation of the saturation effect.



EPLP_1 represents an enzyme complex with PLP bound at the active site (site 1) and EPLP_2 represents an enzyme complex with PLP bound at a second site distinct from the active site (site 2). EPLP_1 is inactive, EPLP_2 is fully active and $\text{EPLP}_1\text{PLP}_2$ cannot be formed. E_t and E represent the total enzyme concentration and free enzyme concentration respectively. It is also assumed that the bound PLP concentration is always an insignificant proportion of the total PLP concentration.

Let

$$K_1 = \frac{(\text{E})(\text{PLP})}{(\text{EPLP}_1)}$$

$$K_2 = \frac{(\text{E})(\text{PLP})}{(\text{EPLP}_2)}$$

$$\text{E}_t = (\text{E}) + (\text{EPLP}_1) + (\text{EPLP}_2)$$

Then % inactivation

$$= \frac{(EPLP_1)}{E_t} \times 100$$

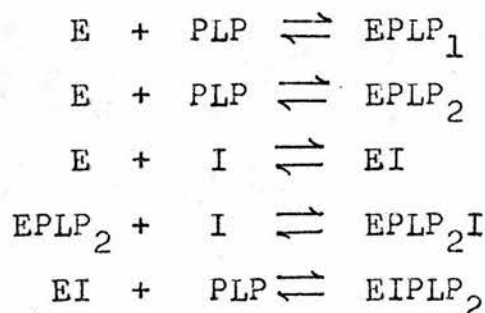
$$= \frac{(E)(PLP)}{K_1} \times 100$$

$$\frac{(E) + \frac{(E)(PLP)}{K_1} + \frac{(E)(PLP)}{K_2}}{K_1}$$

Dividing throughout by $\frac{(E)(PLP)}{K_1}$, and presenting in reciprocal form, gives,

$$100/\% \text{ inactivation} = \frac{K_1}{(PLP)} \left[1 + \frac{K_1}{K_2} \right] \quad (1)$$

If a ligand, I, is competitive with PLP binding at site 1 the above scheme can be expanded to include the following equilibria.



$EPLP_2I$ and $EIPLP_2$ are identical.

$$\text{Let } K_I = \frac{(E)(I)}{(EI)}$$

$$K'_I = \frac{(EPLP_2)(I)}{(EPLP_2I)}$$

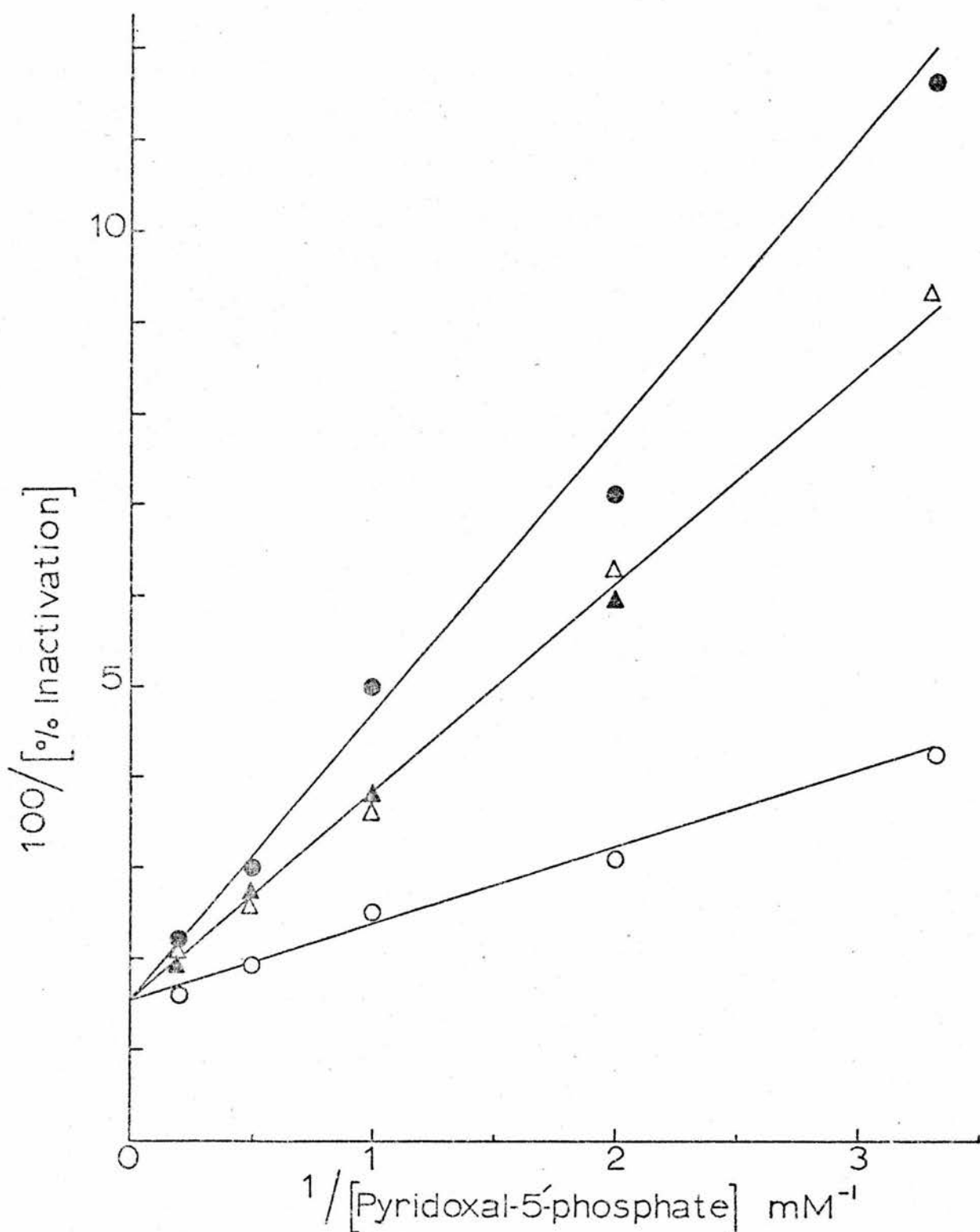


Fig.5-6. The effect of inhibitors on the reversible inactivation of LADH by PLP. Double reciprocal plot of % inactivation versus PLP concentration. Enzyme, 2.0-3.0 μM; phosphate buffer, pH 8.0 (ionic strength maintained at 0.1 by varying the phosphate concentration); ○, without protecting ligand; Δ, + BPCA (33 μM); ●, + AMP (65 μM); Δ, + decanoate (77 μM).

Now let $K_I = \frac{1}{\alpha} K'_I$ where α is a constant and a measure of the interaction between PLP at site 2 and I at site 1.

Thus

$$K_I = \frac{1}{\alpha} \frac{(EPLP_2)(I)}{(EPLP_2I)}$$

And

$$E_t = E + EPLP_1 + EPLP_2 + EI + EPLP_2I$$

Using the same procedure that was used to obtain equation 1 the following equation is derived.

$$100\% \text{ Inactivation} = \frac{K_1}{(PLP)} \left[1 + \frac{(I)}{K_I} \right] + \left[1 + \frac{K_1}{K_2} + K_1 \cdot \frac{(I)}{\alpha K_2 K_I} \right] (2)$$

Thus if α is large only the slope will appear to change and strict competition will be observed. The equilibrium value of % inactivation at different PLP concentrations in phosphate buffer pH 8.0, ionic strength 0.1 and 23.5°C was determined in the absence and presence of inhibitors of LADH. The results are presented as double reciprocal plots. **Inhibition constants** for the inhibitors were calculated from the ratio of slopes = (1 + inhibitor concentration / inhibition constant). The values obtained are shown in table 5-1. They were obtained at a higher pH than those by other methods and are in general smaller.

AMP is strictly competitive with PLP as shown in fig. 5-6. In the above scheme this would imply that AMP was competitive with PLP at both sites. Decanoate is also strictly competitive (fig. 5-6). Decanoate binds at the zinc atom thought to be at the active centre (Sigman, 1967) and competition may represent repulsion between the

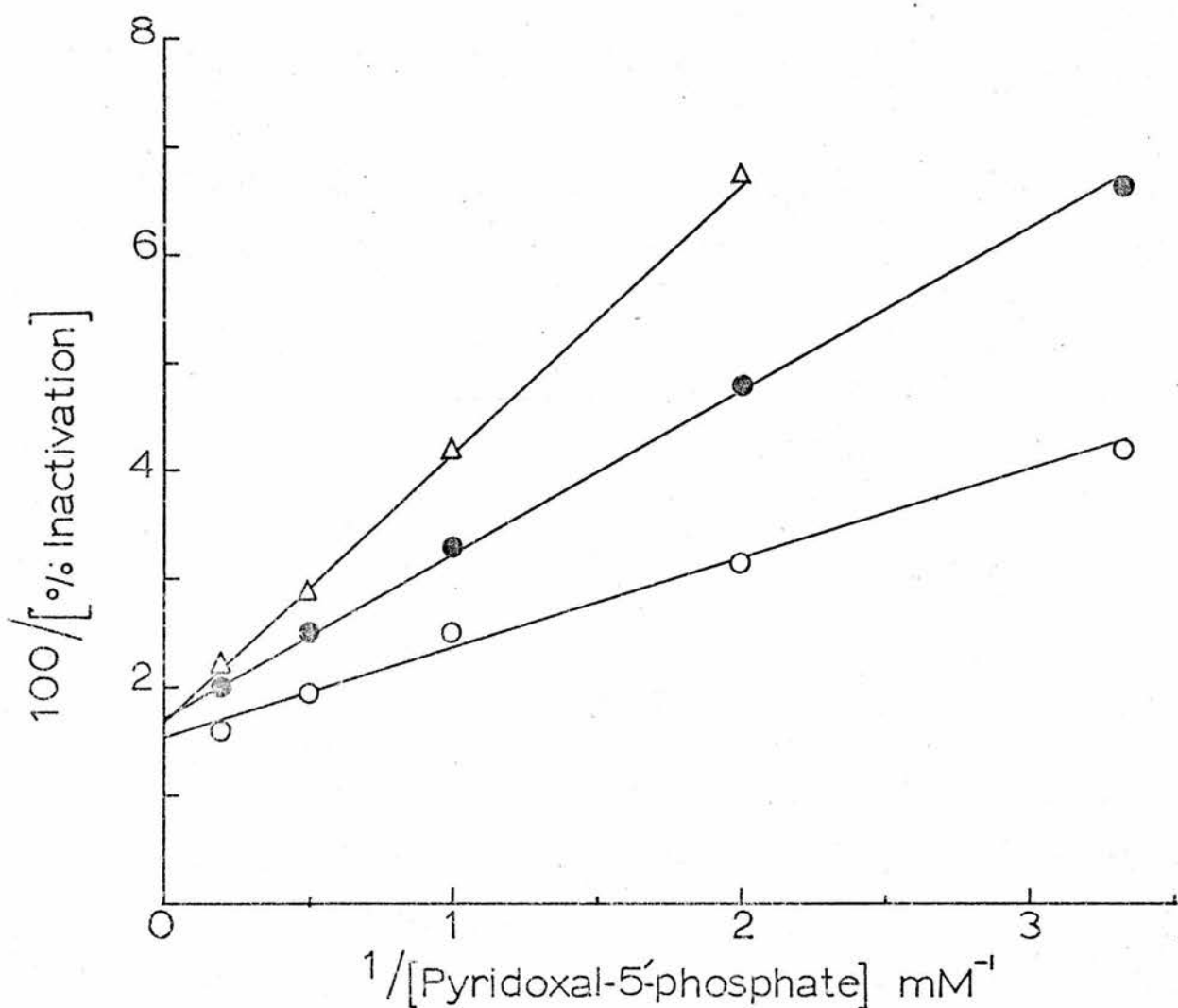


Fig.5-7. Double reciprocal plot showing the effect of adenosine and chloride on the reversible inactivation of LADH by PLP. Enzyme, 2.0-3.0 μM ; phosphate buffer; pH 8.0 (ionic strength maintained at 0.1 by varying the phosphate concentration); ○, without protecting ligand; ●, + chloride (15mM); Δ, + adenosine (6.8mM).

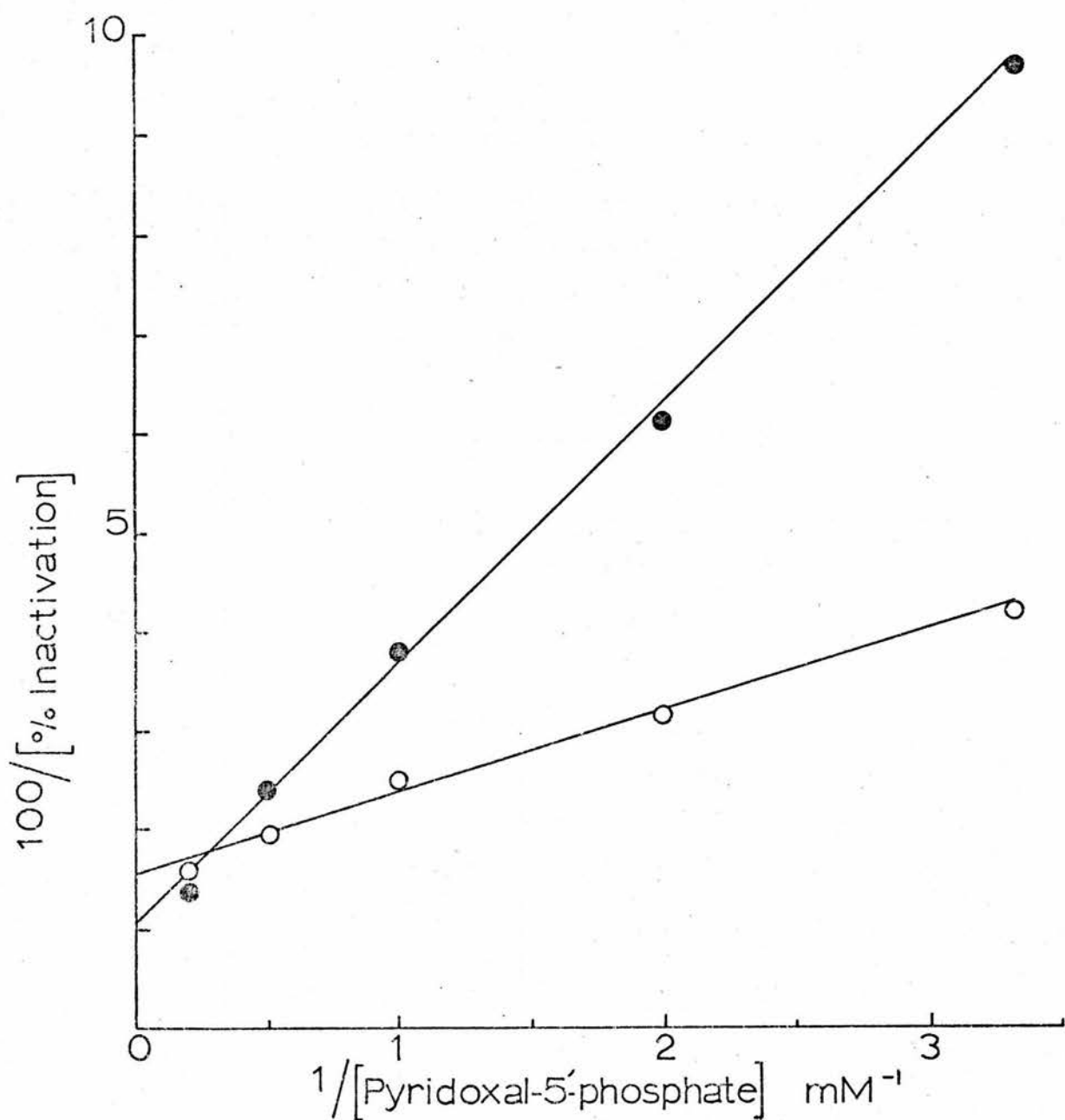


Fig.5-8. Double reciprocal plot showing the effect of ADP-ribose on the reversible inactivation of LADH by PLP. Enzyme, $2.0\text{-}3.0 \mu\text{M}$; phosphate buffer, pH 8.0, (ionic strength maintained at 0.1 by varying the phosphate concentration, o, without ADP-ribose; •, + ADP-ribose ($9.0 \mu\text{M}$)).

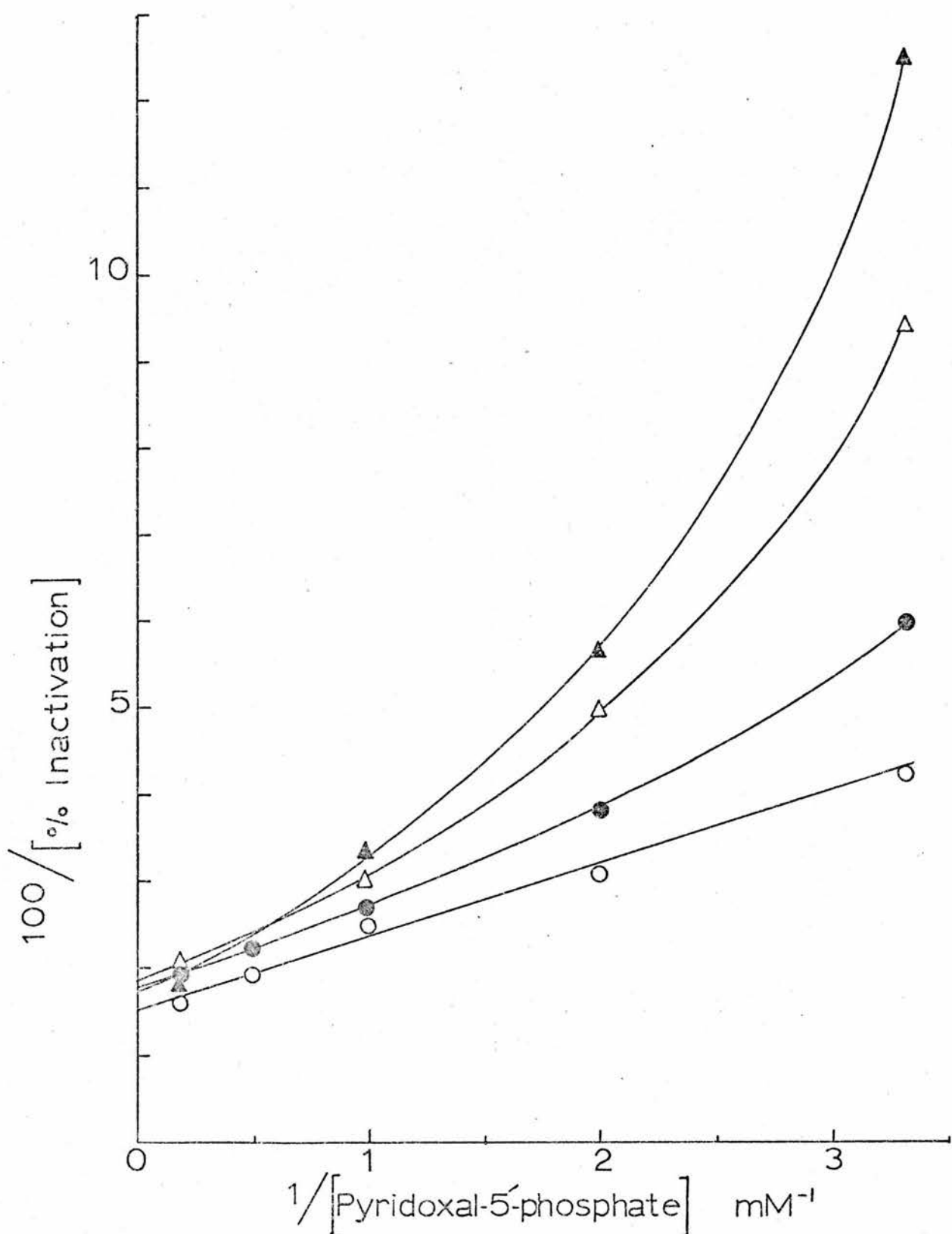


Fig.5-9. Double reciprocal plot showing the effect of neutral zinc binding ligands on the reversible inactivation of LADH by PLP. Enzyme, 2.0-3.0 μ M; phosphate buffer, pH 8.0 (ionic strength maintained at 0.1 by varying the phosphate concentration); o, without protecting ligand; •, + orthophenanthroline (12 μ M); Δ, + orthophenanthroline (28 μ M); ▲, + imidazole (30mM).

negatively charged phosphate group of PLP and the carboxylate group of decanoate. Steric interference is unlikely since decanoate forms a ternary complex with the enzyme and DPN^+ (Winer and Theorell, 1960). 4-Biphenyl carboxylic acid is strictly competitive (fig. 5-6) and since it does not bind at the zinc atom (Sigman, 1967) it is presumably competing with PLP at the active centre lysine. Adenosine and chloride were not strictly competitive (fig. 5-7). The changed intercept indicates that weak LADH-adenosine-PLP and LADH-chloride-PLP complexes can form. The increase in intercept in the presence of adenosine may be due to the formation of EPLP_2 -adenosine rather than an EPLP_1 -adenosine complex. Competition by adenosine with PLP at the AMP binding lysine must be due to steric interference. Since adenosine is a neutral molecule interference with PLP binding at the second site may be slight. ADP-ribose apparently increased the extent of inactivation at high PLP concentration but protected competitively at lower concentrations (fig. 5-8). This would indicate that there is more than one ADP-ribose binding site per subunit. Protection by the neutral zinc binding ligands orthophenanthroline and imidazole is complex as shown in fig. 5-9. At high PLP concentrations these ligands gave weak protection. However, protection increases in a non-linear manner when the PLP concentration is decreased.

The effect of imidazole on the protection of LADH from PLP by some of the above inhibitors was investigated by comparing the protection by the inhibitor at one PLP

TABLE 5-2

The effect of Imidazole on the protection of LADH by inhibitors from reversible inactivation by PLP.

Inactivations were performed at 23.5°C in phosphate buffer, pH 8.0 at constant ionic strength 0.1; PLP; 3.72mM; enzyme, 5 μ N. The concentration of protecting inhibitor was about ten times the dissociation constant for the enzyme inhibitor binary complex.

Inhibitor	Concentration μ M	% Inactivation	% Inactivation in presence of 33.4mM* Imi- dazole
-	-	57	-
-	-	-	58
AMP	550	5	7
ADP-ribose	200	9	20
BPCA	380	1	22
Decanoate	730	30	52
Triiodothyro- acetic acid	20	30	42

* This refers to the concentration of the base form of imidazole.

concentration in the presence and absence of excess imidazole. The results of these experiments are shown in table 5-2. Decanoate protection is weakened as expected since decanoate and imidazole both bind at the active centre zinc. However, BPCA which does not bind to zinc (Sigman, 1967) protects much more weakly in the presence of imidazole. AMP protection is unaltered whereas ADP-ribose protection is appreciably weakened although both compounds bind independently of 2,2'-bipyridyl (Sigman, 1967). Orthophenanthroline protection is not significantly affected. This is as expected since imidazole and orthophenanthroline seem to behave in a similar fashion. Triiodothyroacetic acid which restricts binding of metal chelators to zinc in LADH (McCarthy and Lovenberg, 1969) has its protection significantly diminished by imidazole.

The effect of inhibitors on the number of PLP residues incorporated into the reduced enzyme and the residual activity.

Saturation of the LADH with DPNH, AMP, chloride or bromide completely prevented inactivation and gave rise to an enhancement of activity. AMP and the halides also promoted greater incorporation of PLP groups into the enzyme. These results and others discussed below are shown in table 5-3.

The other ligands had no significant effect on the number of PLP groups incorporated despite the fact that some are protecting the enzyme from inactivation and are presumably shielding the lysine residues necessary for

TABLE 5-3

The effect of inhibitors of LADH on the number of PLP groups incorporated into the reduced PLP-LADH complex and on the residual activity.

(1) Inhibitor	(PLP) mM	Number of PLP groups incorpor- ated per subunit	Residual acti- vity (%)
-	-	-	91
-	7.13	5.5	41
AMP (40mM)	7.13	6.2	135
O.P.*(1.8mM)	7.13	5.9	32
Chloride (1M)	7.13	8.3	107
Bromide (1M)	7.13	7.4	120
(2) -	-	-	98
-	7.13	4.1	25
AMP (40mM)	-	-	98
AMP (40mM)	7.13	5.2	132
(3) -	10.0	4.5	29
BPCA (13mM)	10.0	4.9	16
Decanoate (3.8mM)	10.0	5.2	75
O.P.*(0.28mM)	10.0	4.9	45
(4) -	10.0	4.8	30
AMP (80mM)	20.0	7.2	125
(5) Imidazole (50mM)	10.0	4.8	47
O.P.*(2mM)	10.0	4.8	24
Bromide (1M)	10.0	7.0	100

TABLE 5-3 (contd.)

Inhibitor	(PLP) mM	Number of PLP groups incorpor- ated per subunit	Residual acti- vity (%)
(6) S.D.S. Den- atured LADH	25.0	7.8	-
Carboxymethyl- LADH	25.0	7.8	-
-	25.0	7.1	23

Concentrations of LADH before reduction in above experiments were as follows:-

(1) 13.5 μM : (2) 16.2 μM : (3) 15.0 μM : (4) 30.5 μM
 (5) 15.2 μM : (6) 15.3 μM .

*OP: orthophenanthroline

activity. Denaturation of LADH in 0.075% sodium dodecylsulphate for two hours before diluting into a solution of PLP had no appreciable effect on the number of lysines reacting. Carboxymethylated enzyme behaves similarly to native enzyme in the reactivity of its lysines towards PLP.

The number of lysine residues per subunit of LADH essential for activity

It has been shown that AMP binds competitively with PLP and protects LADH from inactivation by PLP. The number of essential lysine residues protected by AMP per subunit was therefore investigated using AMP and DPNH to protect against inactivation. Since AMP increased the incorporation of PLP into LADH the following method was used. AMP-LADH complex was formed by adding excess AMP and reduced in the presence of PLP. Excess reagents were dialysed away and the activity and number of bound PLP groups of the reduced enzyme determined. The reduced enzyme was then reduced a second time in the presence of PLP and dialysed. The activity and number of bound PLP groups after this second reduction were determined. The difference between the number of bound PLP residues after the first and second reductions was assumed to represent the number of lysines protected by AMP.

DPNH did not promote additional incorporation of PLP into LADH so a direct comparison was made between the number of bound PLP groups in reduced PLP-LADH-DPNH complex and reduced PLP-LADH. The results of these experiments are outlined in table 5-4. A minor degree of

TABLE 5-4

Estimation of the number of lysine residues of LADH protected from reaction with PLP by AMP and DPNH

(a) Protection by AMP: LADH completely in the form

LADH-AMP was reduced with sodium borohydride in the presence of PLP. Reagents were then removed by dialysis and the reduced PLP-enzyme reduced again in the presence of PLP and dialysed. The number of PLP groups bound and the relative enzyme activity was determined after each reduction.

Reduction Step	+ AMP		- AMP	
	Number of PLP groups bound per subunit	Activity (%)	Number of PLP groups bound per subunit	Activity (%)
(1) First	5.8	111	4.8	26
Second	7.3	20	6.4	1
Third	7.5	1	7.3	0
(2) First	8.3	125	-	-
Second	9.3	26		

(b) Protection by DPNH: Two enzyme solutions one containing DPNH and one without were reduced in the presence of PLP and dialysed. The difference between the number of PLP groups bound was assumed to be the same as the number of lysines protected by DPNH.

	+ DPNH		- DPNH	
	(1)	(2)	(3)	
(3) First	6.3	112	7.1	23
Experiment	(1)	(2)	(3)	
Number of lysines protected per subunit	1.5	1.0	0.8	
Activity loss (%)	82	79	79	

TABLE 5-4 (contd.)

The following ratios of PLP concentration to enzyme concentration were used in these experiments:-

(1) 330; (2) 1,200; (3) 1,650.

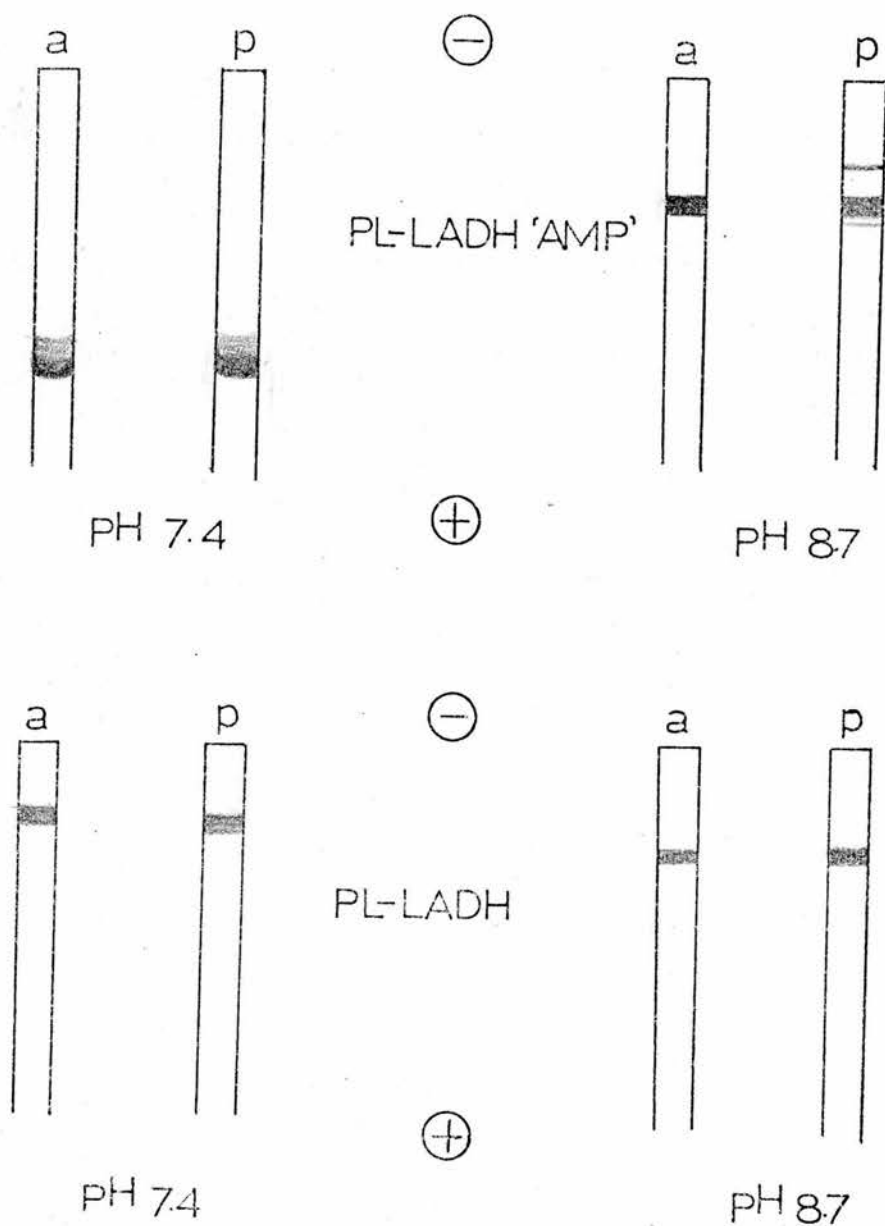


Fig.5-10. Polyacrylamide gel electrophoresis of LADH covalently labelled with PLP with and without the presence of saturating concentration of AMP (PLP-LADH and PLP-LADH'AMP').

15% Gels prepared in phosphate buffer, pH 7.4, ionic strength 0.02, and 0.3M borate buffer, pH 8.7, were used. a, indicates that the gel was stained for enzyme activity. p, indicates that the gel was stained for protein. PLP-LADH had 7.3 PLP residues incorporated per subunit and a residual activity of 23%. PLP-LADH'AMP' had 8.3 PLP residues incorporated per subunit and a residual activity of 125%.

incorporation of PLP residues probably occurs at lysines not affecting activity during second and successive reductions, thus giving slightly inflated values for the number of lysines protected. A large excess of PLP during the first reduction will help to reduce this error. Inspecting the results of experiments (1) and (2) (table 5-4), it can be seen that experiment (2) in which there was a much greater excess of PLP than in experiment (1), gives a significantly smaller value for the number of lysines protected. Experiments (2) and (3) show that modification of 1.0 - 0.8 lysines per subunit resulted in 80% inactivation and it would be reasonable to suggest that there is one lysine per subunit which is in the coenzyme binding site. An important observation was that repeated reduction in the presence of PLP led to complete inactivation and indicates that PLP-LADH does not have residual activity.

Electrophoresis of reduced PLP-LADH

Reduced PLP-LADH with 7.3 PLP groups incorporated per subunit and a residual activity of 23% and PLP-LADH with 8.3 PLP groups incorporated per subunit and an enhanced activity of 125% (produced by modifying the enzyme AMP complex) were compared by polyacrylamide gel electrophoresis at pH 7.4 and pH 8.7. Both enzyme migrated towards the anode, the opposite direction to native enzyme. The mobility of the modified enzyme was also considerably greater than native enzyme. At both pH values and for both inactivated and activated enzymes a single major band was obtained on staining for activity and protein. As

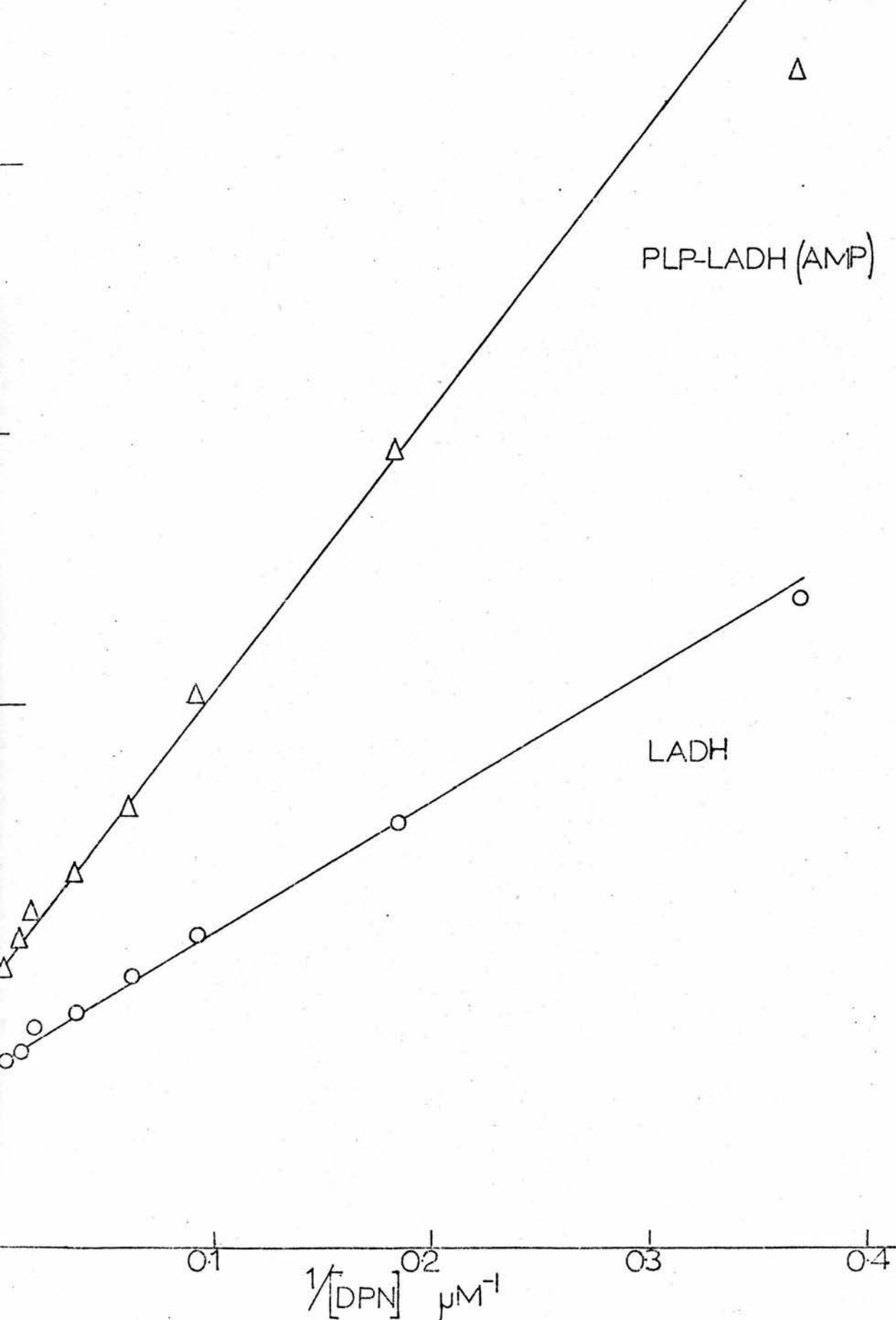


Fig.5-11. Kinetics with constant ethanol concentration (8.25mM) and varied DPN^+ concentration catalysed by native LADH (o) and PLP-LADH'AMP' (Δ). Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C.

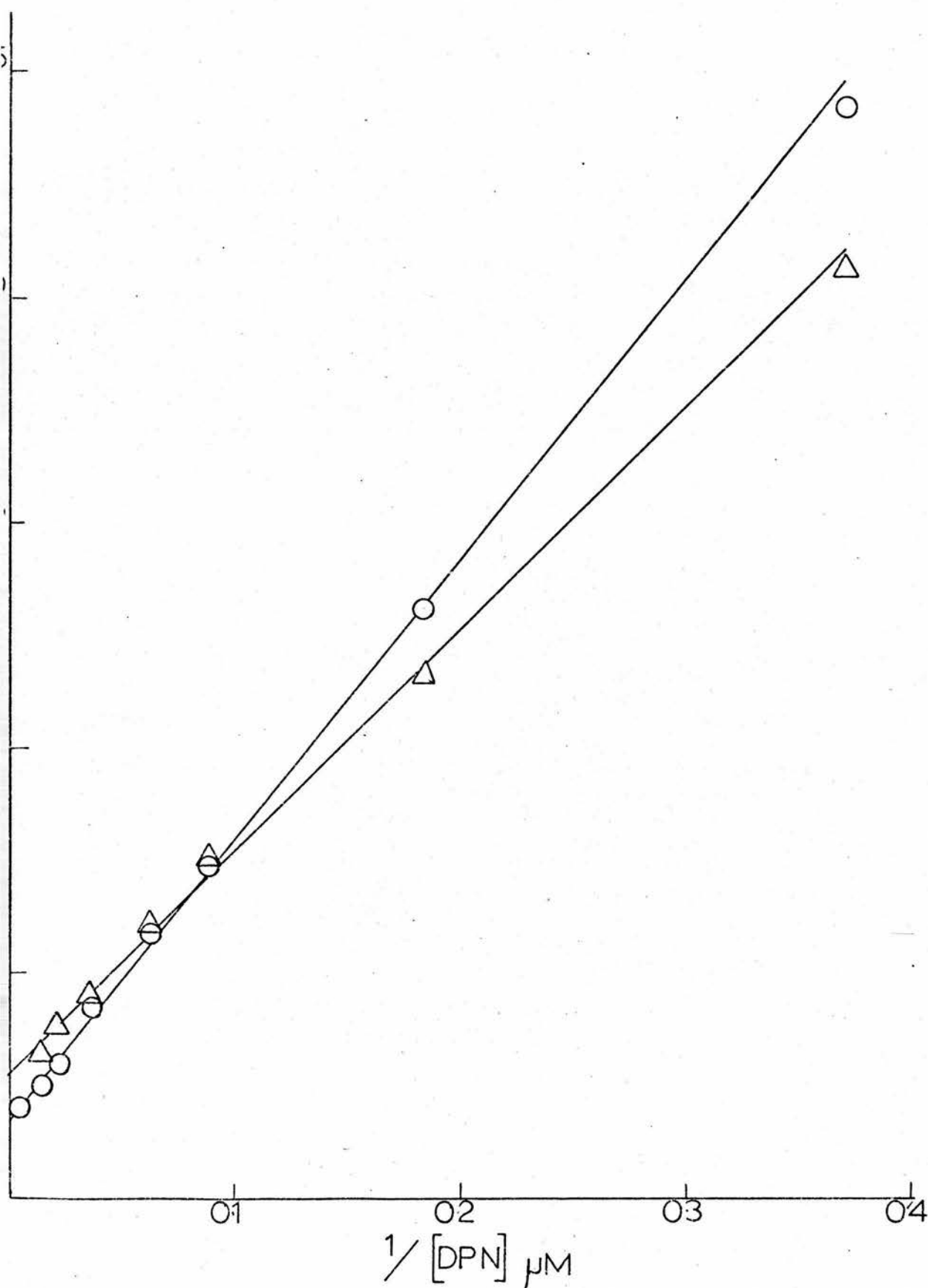


Fig.5-12. Kinetics with constant ethanol concentration (3.25mM) and varied DPN^+ concentration catalysed by native LADH (Δ) and PLP-LADH'AMP' (\circ). Buffer, 10.0mM glycine-NaOH plus phosphate, pH 10.0, ionic strength 0.1; 23.5°C.

can be seen in fig. 5-10 the activity and protein bands occur at the same position. A minor less acidic band appeared in the protein stain of PLP-LADH 'AMP' at pH 8.7. The above results indicate that all the enzyme molecules in a given preparation are probably substituted to the same extent. This means that in PLP-LADH with 20% activity although one molecule may be completely inactivated and another have its activity enhanced, both molecules have the same number of PLP groups incorporated.

Kinetic experiments with PLP-LADH 'AMP'

The reduction of DPN^+ by ethanol and native LADH or PLP-LADH 'AMP' was compared at pH 7.1 and pH 10.0 (fig. 5-11 and fig. 5-12). The binding of AMP and ortho-phenanthroline to the modified enzyme at pH 7.1 was also studied. The results are presented in table 5-5. At both pH 7.1 and 10.0 V_{max} and K_m were altered. V_{max} was increased by 64% at pH 10 and decreased by 33% at pH 7.1. At both pH 7.1 and pH 10 K_m was increased indicating a weakening of coenzyme binding. At pH 7.1 AMP binding was weakened by more than 2-fold while orthophenanthroline binding was essentially the same. The pKs of the ionizing groups of PLP residues in the reduced Schiff bases might be expected to be similar to those of pyridoxamine-5'-phosphate (i.e. 3.54 (phenolic), 6.2 (phosphate), 8.21 (pyridinium nitrogen). The total charge of a PLP residue will therefore change considerably from pH 7.1 to pH 10. Thus changes in enzymic activity on modification of LADH with PLP might be expected to be pH dependent.

TABLE 5-5

Comparison between the kinetics of DPN^+ reduction by ethanol catalysed by LADH and PLP-LADH 'AMP'* at pH 7.1 and pH 10.

(a) pH 7.1. Phosphate buffer, ionic strength 0.1, 23.5°C .

	<u>LADH</u>	<u>PLP-LADH 'AMP'</u>
$K_m \text{DPN}^+$ (Ethanol, 8.25mM)	$7.6 \mu\text{M}$	$10.4 \mu\text{M}$
V_{max}	3.0 sec^{-1}	2.0 sec^{-1}
$K_{E, \text{AMP}}$	$69 \mu\text{M}$	$164 \mu\text{M}$
$K_{E, \text{OP}}$	$6.2 \mu\text{M}$	$5.3 \mu\text{M}$

(b) pH 10.0, 10mM glycine NaOH plus phosphate to give an ionic strength of 0.1, 23.5°C .

$K_m \text{DPN}^+$ (Ethanol, 8.25mM)	$17 \mu\text{M}$	$34 \mu\text{M}$
V_{max}	3.6 sec^{-1}	5.9 sec^{-1}

* PLP-LADH 'AMP' refers to LADH which had 7.2 PLP groups incorporated when reduced in the presence of PLP and a saturating concentration of AMP.

The effect of PLP on the rate of inactivation of LADH by iodoacetate

A preliminary experiment to investigate the possibility of interaction between iodoacetate and PLP was carried out. A mixture of 17.9mM iodoacetate and 1.67mM PLP at pH 7.4 did not generate a difference spectrum between 260nm and 600nm after incubation for two hours. It was therefore concluded that reaction between the two compounds was unlikely. A double reciprocal plot showing protection of LADH from iodoacetate by PLP is given in fig. 5-13. The enzyme was added to a solution of iodoacetate and PLP to initiate the inactivation. Since the formation of PLP-LADH requires ten to fifteen minutes the plots of log activity with time at different iodoacetate concentrations were not linear until the PLP-LADH had reached equilibrium concentrations. It is not obvious why PLP and iodoacetate did not give strictly competitive kinetics as iodoacetate and AMP are strictly competitive and AMP and PLP are strictly competitive. The dissociation constant of PLP-LADH calculated from the ratio of slopes = $1 + (\text{PLP concentration} / \text{dissociation constant})$ was 2.9mM when the PLP concentration was 5mM. However, a Dixon plot of half time of inactivation against PLP concentration at constant iodoacetate concentration of 2.688mM and constant ionic strength, 0.1, was not linear but concave-down. This is to be expected if PLP is unable to saturate the lysine residue at the active centre which is perhaps also the iodoacetate binding site.

Discussion

The residual activity of LADH modified by formation of Schiff bases between PLP and lysine residues in the enzyme does not seem to be a property of enzyme with the active centre lysine modified since successive reductions in the presence of PLP leads to complete inactivation. It is proposed that the residual activity of modified enzyme is due to a mixture of inactive enzyme in which the active centre lysine is modified, and fully active enzyme (or enzyme with enhanced activity) in which lysines other than the active centre lysine are modified. Inactivation is probably due to repulsion between the negatively charged groups of PLP bound at the active centre lysine and the phosphate group of the AMP moiety of the coenzyme preventing coenzyme from binding. It is postulated that the failure of increasing PLP concentration to lead to complete inactivation is due to protection of the active centre lysine by PLP binding at a second site. This site could be near enough the active lysine for electrostatic repulsion between negatively charged groups of the molecules to be too strong for both molecules to bind simultaneously. Because successive reductions in the presence of PLP led to complete inactivation this second site can not be a lysine residue, otherwise covalent labelling of the second site with PLP would give permanent protection. An arginine residue or a zinc atom are possible positively charged alternative binding sites. Benzaldehyde which would not be expected to protect in this manner inactivated LADH to a greater extent than PLP.

Any chemical modification of a protein will lead to

some degree of conformational change. The substitution of PLP with its three ionizing groups for the single positive charge of the ϵ -amino group of lysine will result in considerable changes in the charge balance on the enzyme surface giving rise to conformational changes. These changes may result in the active centre lysine being sterically hindered from binding PLP, or negatively charged amino acid residues may be moved close enough to interfere electrostatically. However, this and similar explanations of the saturation effect do not explain why successive covalent labellings result in complete inactivation.

The pH dependence of the extent of inactivation of LADH by PLP, which shows a maximum between pH 8 and pH 9, is typical of the pH dependence of imine formation between amino acids and pyridoxal derivatives. The pH dependence for imine formation for pyridoxal with valine and glycine shows a maximum for the equilibrium constant at approximately pH 9 (Metzler, 1957).

The active centre lysine seems to be a relatively strong binding site for PLP since fig. 5-5 shows that the enzyme is 70% inactivated when only 5 PLP groups have been incorporated per subunit compared to 30 potential lysine binding sites per subunit. The phosphate group of PLP may bind similarly to the phosphate of AMP thus facilitating Schiff base formation with the active centre lysine. This mechanism of selectivity has been used to explain the modification of lysine by PLP at one of the two phosphate binding sites of rabbit muscle aldolase which led to 70% loss of activity when only 6 moles of PLP were

incorporated per mole of enzyme (Shapiro et al., 1968).

The Schiff base between PLP and the active centre lysine may be stabilized by interaction between the phosphate group of PLP and the active centre zinc atom. Strict competition between decanoate and PLP indicates that these sites are close enough for electrostatic repulsion between anions bound at the two sites to be strong. Reynolds et al., (1970) have also shown that the zinc atom plays an important part in stabilizing the binding of AMP.

Experiments designed to find the effect of ionic strength on the binding of PLP and benzaldehyde, using phosphate to vary the ionic strength, indicated that phosphate may bind at the active centre lysine. It has been shown previously that phosphate is competitive with TPNH (Dalziel and Dickinson, 1965) and Reynolds (1970) found that it is very difficult to obtain LADH free from phosphate by dialysis.

Protection by AMP was strictly competitive with PLP and one lysine per active centre was protected. This lysine has therefore been referred to as the active centre lysine. If the scheme described in the Results section, involving the PLP binding site at which bound PLP protects the active centre lysine, is correct AMP must be competitive with PLP at both the sites, otherwise in equation (2) α will be finite and the intercept will be altered. Lysine has been postulated to be the binding site for AMP in pig kidney fructose 1,6-diphosphatase because PLP desensitises the enzyme to AMP inhibition.

(Marcus and Hubert, 1968). Johnson and Deal (1969) discovered that the rate of specific binding of 2 to 4 moles of PLP per mole of rabbit muscle pyruvate kinase was decreased by certain phosphate containing metabolites, including ADP and ATP.

ADP-ribose is competitive with PLP but also increases the extent of inactivation at infinite PLP concentration. Weiner (1969) has shown that an analogue of DPN^+ binds at sites other than the active centre. ADP-ribose perhaps behaves similarly and interferes with PLP binding at the non-lysine PLP binding site thus increasing the affinity of the active centre lysine for PLP.

BPCA is strictly competitive with PLP. Since BPCA does not bind at the active centre zinc atom (Sigman, 1967) it may be binding by the carboxylate group to the active centre lysine. However, excess imidazole was found to considerably decrease the protection by BPCA, from PLP inactivation. This would indicate that the active centre zinc atom is also involved in BPCA binding. Reynolds, et al., (1970) found that at pH 7.4 excess imidazole weakened BPCA binding to a greater extent than AMP binding. Thus it may be that the carboxylate group of BPCA is bound by the active centre lysine, but nearer the active centre zinc atom than the phosphate group of AMP.

Strict competition between decanoate bound at the active centre zinc and PLP at the active centre lysine indicates that the postulated non-lysine PLP binding site must be near enough the zinc for decanoate to prevent PLP

binding there, presumably by electrostatic repulsion, although a conformational change induced by decanoate can not be ruled out. It is not unlikely that an anion binding at the active centre zinc would be competitive with PLP binding at the active centre lysine since decanoate and AMP are competitive and evidence exists to suggest that the AMP binding site and the active centre zinc are close enough for electrostatic interaction to help to stabilize AMP binding (Reynolds et al., 1970).

Both chloride and adenosine form complexes with the enzyme in the presence of infinite concentrations of PLP. Adenosine being a neutral molecule may not interfere with PLP binding at the non-lysine site. In this case in equation (2), α is finite, and the intercept of the double reciprocal plot is increased. The fairly good agreement between K_I for adenosine obtained by steady state kinetics and by PLP inactivation (table 5-1) indicates that adenosine is competitive with PLP at the active centre lysine.

Chloride enhanced the incorporation of PLP into LADH, which suggests that it may produce a conformational change in the enzyme. Other evidence for a chloride induced conformational change is the increased rate of breakdown of ER in the presence of chloride (Theorell et al., 1955) and the increased ease of zinc exchange in the presence of chloride (Drum, 1970). Thus, although chloride is probably competitive with PLP at the active centre lysine it may not affect PLP binding at the non-lysine site to the same degree, or a conformational change may be altering the ratio of affinities of the active centre lysine

and non-lysine sites for PLP. In both cases the protection of the active centre lysine by PLP at the non-lysine site will be increased. It is also probable that chloride is binding at sites in addition to those discussed above.

The effects of imidazole and orthophenanthroline on the inactivation of LADH by PLP are very similar, although complex. At concentrations at which they bind strongly to the active centre zinc atom little protection is seen. The binding of PLP may be stabilized by interaction of the negatively charged groups on the molecule with the active centre zinc atom and the binding of a neutral ligand at the zinc, which does not sterically interfere with the binding of molecules such as AMP and ADP-ribose at the active centre lysine, should not decrease, but perhaps increase this interaction. It has been shown that orthophenanthroline does not displace AMP from the enzyme (Yonetani and Theorell, 1964) and ADP-ribose does not displace 2,2'-bipyridyl (Sigman, 1967). Imidazole and orthophenanthroline are perhaps protecting by binding at a site other than the active centre zinc atom, possibly by causing a conformational change. Imidazole has previously been conjectured to cause a conformational change in LADH due to binding of imidazole at a weaker binding site than the active centre zinc atom (Reynolds et al., 1970). The concave-up plots shown in fig. 5-9 may indicate that the binding of more than one PLP molecule per subunit is implicated in inactivation of LADH in the presence of excess neutral zinc binding ligand. As discussed above

these ligands may produce conformational changes which protect the active centre lysine. However, the binding of PLP elsewhere on LADH may alter the enzyme conformation by changing the charge balance on the enzyme surface and this may counteract the protection by the zinc binding ligands exposing the active centre lysine to PLP. The inactivation by PLP would thus appear to be co-operative and give rise to concave-up double reciprocal plots.

A maximum of about 11 PLP groups were incorporated per subunit of LADH, which represents modification of 30% of the lysines in the enzyme. It is reasonable to assume that most of the charged amino acid functional groups are exposed at the surface of the enzyme. X-ray analysis of 10 globular proteins has provided evidence that the majority of hydrophobic side chains are located in the core of the protein, whereas the majority of polar and electrically charged side chains prefer the surface of the protein (Perutz, 1969). Plapp (1970) has shown that 50 of the 60 lysines of LADH reacted with methylpicolinimidate, which suggests that most of the lysines in the enzyme are exposed to the medium. Examination of the primary sequence of LADH (Jörnvall, 1970 c) gives a hint of how the number of PLP groups bound might be limited. Defining close proximity as separation by at the most two residues in the sequence, then the lysines fall roughly into three categories:

- (a) Lysines which are in close proximity to an aspartate or glutamate residue will be in an unfavourable environment for binding PLP due to

electrostatic repulsion between the phosphate and phenolic group of PLP and the carboxylate groups of the amino acids. Seventeen of the 30 lysines per subunit are in the category.

(b) Lysines in close proximity to another lysine.

Such a lysine is in a favourable environment for binding PLP until the other lysine is modified by PLP when it will be strongly protected.

Thus only one of such a pair of lysines will be modified. Seven lysines per subunit fall in this category of which half will bind PLP.

(c) Lysines in close proximity to an arginine residue.

A Schiff base between these lysines and PLP will be stabilized by attraction of the phosphate group of PLP to the positively charged guanidinium group.

Four lysines per subunit fall in this category.

In addition to the lysines included in the above categories two lysines are located in hydrophobic regions of the sequence. The sum of available lysines in categories (b) and (c) is 7.5, which compares fairly well with the maximum of about 11 PLP groups found to be incorporated per subunit of LADH. This approach has ignored possible effects due to the zinc atoms in the enzyme (the active centre zinc atom is postulated above to be stabilizing the binding of PLP at the active centre lysine), or the packing of the polypeptide chain in the tertiary structure. However, some support for the validity of this approach comes from the finding that denaturation of LADH in

sodium dodecyl sulphate does not appreciably increase the incorporation of PLP into the enzyme. The number of PLP groups incorporated need not represent the number of lysines binding PLP since all exposed lysines may bind PLP to some extent. However, it may well be an indication of the number of strong PLP binding sites. It is interesting to note that Weiner (1969) using an analogue of DPN^+ revealed that LADH has two classes of binding sites for the analogue: two strong binding sites and 5-6 weak binding sites.

Enzyme which was protected by AMP while being covalently labelled with PLP (referred to as PLP-LADH'AMP') was found to have enhanced activity. This increase in V_{max} was found at pH 10 but not at pH 7.14 and may be due to a pH dependent conformational change. The pK_α of the pyridinium nitrogen of the reduced Schiff base of PLP with lysine is assumed to be similar to the corresponding pK_α for pyridoxamine which is 8.21. The net charge of the incorporated PLP molecules will therefore increase from -2 at pH 7.14 to -3 at pH 10.0 and conformational changes caused by alterations in the charge balance on the enzyme surface may be very different at these two pH values.

The K_m for DPN^+ was increased at both pH values. Since $K_m = \frac{k_2}{k_1}$ at infinite alcohol concentration (see Chapter 1), this is due to either an increase in k_2 , the rate of breakdown of ER, or a decrease in k_1 , the rate of association of EO. Since at pH 10 V_{max} is increased the rate of dissociation of ER must also be increased, which

indicates that the increase in the K_m of DPN^+ is due to an increase in k_2 . The reason why V_{max} is not increased at pH 7.14, despite the weakening of DPNH binding, is not clear. The weakening of DPNH binding is probably due to an effect on the binding of the AMP moiety of the coenzyme since at pH 7.14 binding of AMP to PLP-LADH'AMP' is 2-fold weaker than binding to LADH whereas orthophenanthroline binding is the same.

CHAPTER 6.

The Reaction of Methylpicolinimidate and
2-Methoxy-5-Nitrotropone with the Lysine Residues of LADH

Introduction

It has been discovered (Plapp, 1970) that MP1 reacts with 50 of the 60 lysine residues of LADH resulting in a 19-fold activation of the enzyme when assayed at pH 9.0, with high concentrations of DPN^+ and ethanol. On the evidence of product inhibition studies, Plapp considered that the enzyme mechanism was unchanged and the enhancement of activity was due to faster dissociation of the binary complexes of LADH with DPN^+ and DPNH . He concluded that co-enzyme binding was weakened due to modification of at least one lysine residue near the nicotinamide ring binding site of the coenzyme.

MP1 was thus a useful reagent to test the conclusions reached from the results of modification of the lysines of LADH with PLP. Modification with PLP replaces the positive ϵ -ammonium group of lysine with a negatively charged group whereas picolinimidation preserves the positive charge. It was also decided that modification of lysine resulting in an uncharged residue would usefully supplement the modifications with PLP and MP1. 2-Methoxy-5-nitro tropone was tried as it has been reported to be specific for amino groups under mild conditions, the degree of modification being readily measurable and reversible (Tamaoki et al., 1967).

Experimental

MP1 was synthesised according to the method of Schaefer and Peters (1961). The product distilled at b.p. $109-111^{\circ}\text{C}$ (20mm) and was stored at -14°C . 0.1M solutions of this reagent were prepared by additions of $12.5\mu\text{l}$

MP1 per ml. of solution, assuming a density of 1.1 gm/ml.

The modified enzyme was assayed in 62mM sodium glycinate pH 10, 1.68mM DPN⁺, 550mM ethanol at 23.5°C (assay -2).

Activations were performed in 0.5M N-ethylmorpholine-HCl, pH 8.0 at 23.5°C and allowed to proceed for 3-4 hours. Longer incubation leads to a progressive loss of activity (Plapp, 1970). The activations were initiated by addition of MP1 and followed by measuring the initial velocity of DPN⁺ reduction recorded with a Gilford 2000 recording spectrophotometer. The incubation solution was diluted (26-fold) into 0.5M N-ethylmorpholine-HCl, pH 8.0, to provide a concentration of enzyme suitable for accurate assay.

The concentrations of picolinimidated enzyme and nitro-troponyl enzyme were determined by alkaline hydrolysis and ninhydrin analysis (Frechter and Crestfield, 1965; Moore, 1968). The colour value of 6.3 μ moles of leucine per mg. of enzyme determined previously was used.

Determination of the number of picolinimide groups incorporated into LADH was determined from the difference in absorption at 262nm between LADH and picolinimidated LADH using the molar extinction coefficient of N-butyl-picolinimidine-HCl at 262nm equal to 5,700M⁻¹cm⁻¹ (Benisek and Richards, 1959).

Reactions with MNT were carried out in 0.5M N-ethylmorpholine, pH 8.5, at room temperature using about 50 μ M LADH. Aliquots of the incubation solution were withdrawn at suitable intervals and passed through a column of G25 sephadex (1.4 x 12cm) equilibrated with phosphate buffer

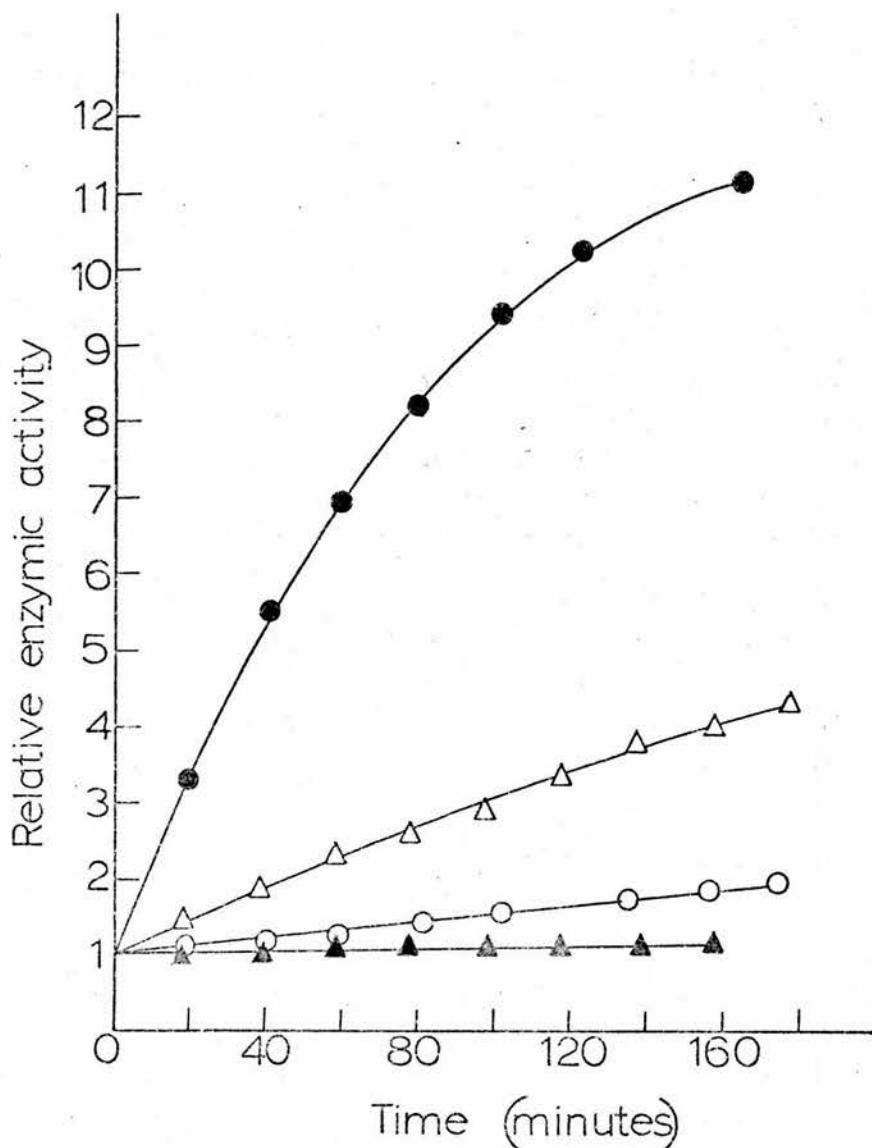


Fig.6-1. Effect of DPNH and AMP on the activation of LADH by MPI. Enzyme, $9.2\mu\text{M}$; MPI, 0.1M ; buffer, 0.5M N-ethylmorpholine HCl, pH 8.0; 23.5°C ; ●, without added ligand; Δ, + AMP (8.76mM); ○, + AMP (36.60mM); ▲, + DPNH (0.52mM). Activity was related to the enzyme activity of the activation solution before addition of MPI at time zero.

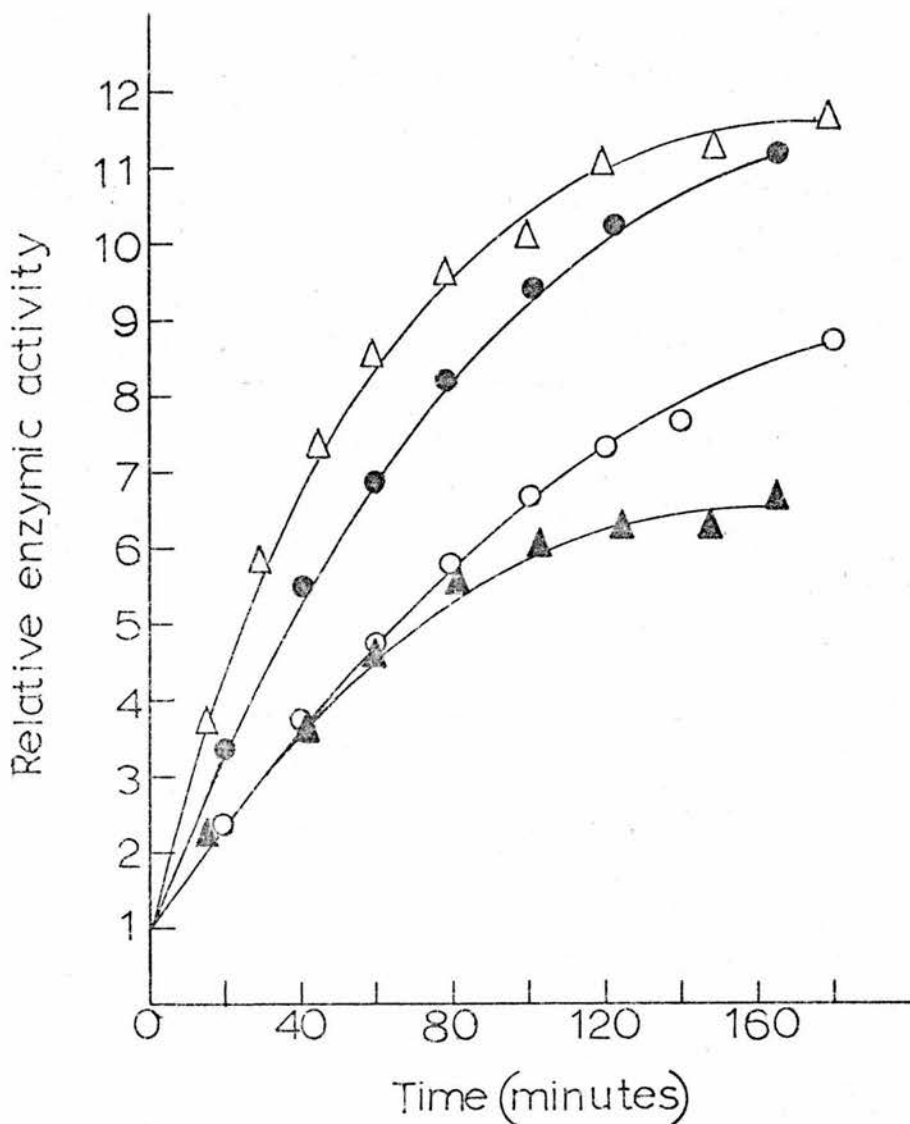


Fig.6-2. The effect of neutral zinc binding ligands on the activation of LADH by MPI. Enzyme, $9.2\mu\text{M}$; MPI, 0.1M ; buffer, 0.5M N-ethylmorpholine HCl, pH 8.0; 23.5°C ; ●, without added ligand; Δ, + imidazole (64.9mM); ○, + 2,2-bipyridyl (25.6mM); ▲, + orthophenanthroline (2.22mM). Activity was related to the enzyme activity of the activation solution before addition of MPI at time zero.

pH 7.4, ionic strength 0.1, to separate the modified protein from excess reagent. The number of nitrotroponyl residues incorporated into LADH was determined spectrophotometrically at 420nm using the millimolar extinction coefficient of 20.7 for α -N-acetyl- ϵ -N-nitrotroponyl lysine (Tamaoki et al., 1967).

Results

Reaction with MP1

Reaction of LADH with MP1 for three hours resulted in an 11-fold activation of enzyme activity assayed at pH10 and a 17-fold increase in activity assayed at pH 9. The pH 9 assay solution contained 85mM $\text{Na}_4\text{P}_2\text{O}_7$, 18mM glycine, 1.68mM DPN^+ and 550mM ethanol.

The effect of coenzyme and inhibitors on the rate of activation

Reaction of the enzyme-DPNH complex with MP1 resulted in a very slight increase in activity (fig. 6-1). This almost complete protection by reduced coenzyme contrasts with the partial protection found by Plapp (1970). Plapp's failure to find complete protection by DPNH was almost certainly due to the fact he had only 75% of the LADH in the binary enzyme-DPNH complex.

When the enzyme was saturated with AMP there was a slow rate of activation, 2-fold in 3 hours (fig. 6-1). This seems to indicate that the major part of the enzyme rate enhancement is due to picolinimidation of the single lysine protected by AMP from reaction with PLP.

BPCA protected to a small extent; a 9-fold activation was achieved in three hours instead of the 11-fold activation in the absence of inhibitor (fig. 6-3).

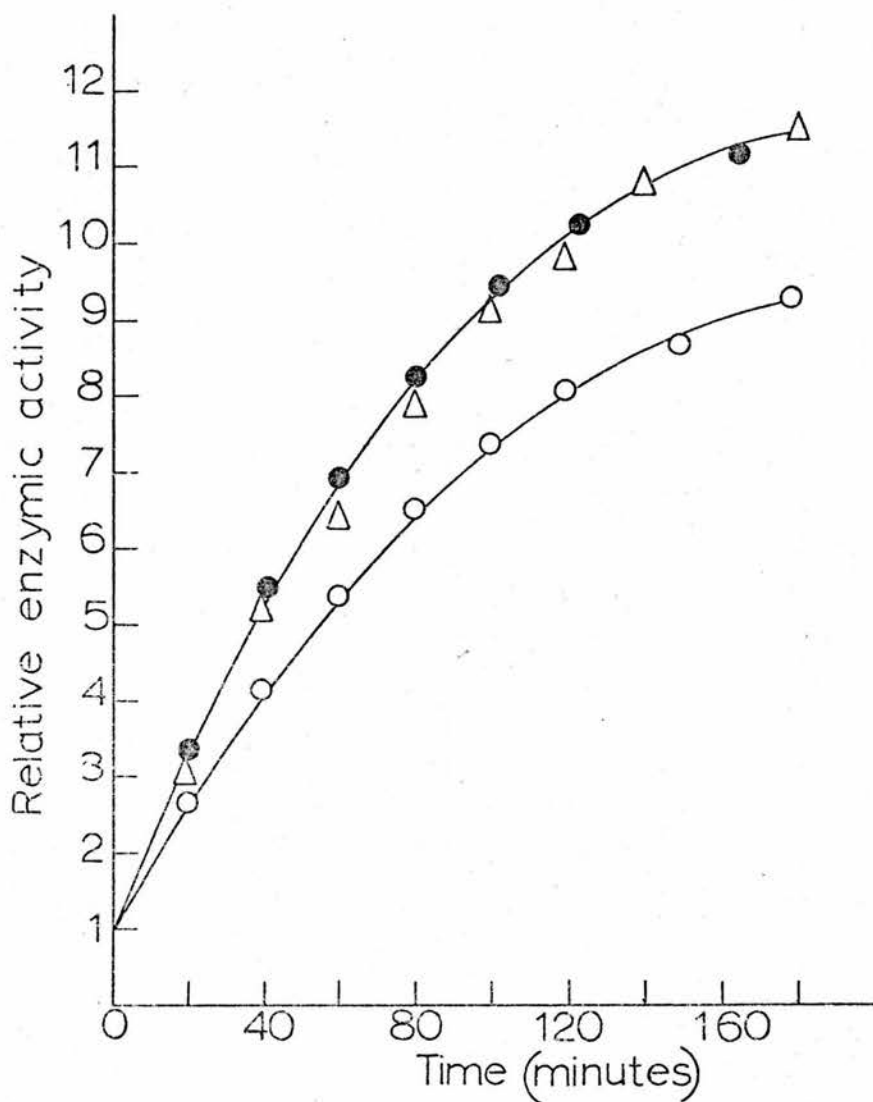


Fig.6-3. The effect of decanoate and 4-biphenylcarboxylic acid on the activation of LADH by MPI. Enzyme, $9.2\mu\text{M}$; MPI, 0.1M ; buffer, 0.5M N-ethylmorpholine HCl, pH 8.0; 23.5°C ; ●, without added ligand; Δ, + decanoate (5.51mM); ○, + 4-biphenylcarboxylic acid (10.1mM). Activity was related to the enzyme activity of the activation solution before addition of MPI at time zero.

Decanoate which binds at the active zinc (Sigman, 1967) produced no effect on the rate of activation (fig. 6-3).

The neutral zinc chelating ligands, orthophenanthroline and 2,2-bipyridyl, however, gave partial protection. In both cases the initial rate of picolinimidation was the same, but picolinimidation of the enzyme orthophenanthroline complex was apparently complete in about two hours when the activity had increased 6-fold (fig. 6-2). Since orthophenanthroline does not remove zinc from native LADH (Drum and Vallee, 1969) this effect may be due to a labilising of zinc in picolinimidated enzyme facilitating its removal by orthophenanthroline, but not by bipyridyl.

Imidazole did not change the final extent of activation, but increased the rate at which activation took place. In the presence of excess imidazole the initial rate of activation was 13-fold per hour compared with 9.5-fold per hour for the control activation (fig. 6-2).

The effect of phosphate on activation of LADH by MP1 was investigated (fig. 6-4). Activations in 0.5M phosphate, pH 8.0, resulted in a maximum activation of 7-fold after 3 hours (fig. 6-4). The rate of activation was accelerated by imidazole from 5-fold per hour to 9-fold per hour, an appreciably larger enhancement than in N-ethylmorpholine-HCl, buffer. Maximum activation in excess imidazole was also 7-fold, achieved in 1.5 hours, after which the activity began to decrease. Activation in 0.5M N-ethylmorpholine-phosphate buffer, pH 8.0, resulted in 6-fold activation after 3 hours. Activation in both 0.1M phosphate and 0.1M

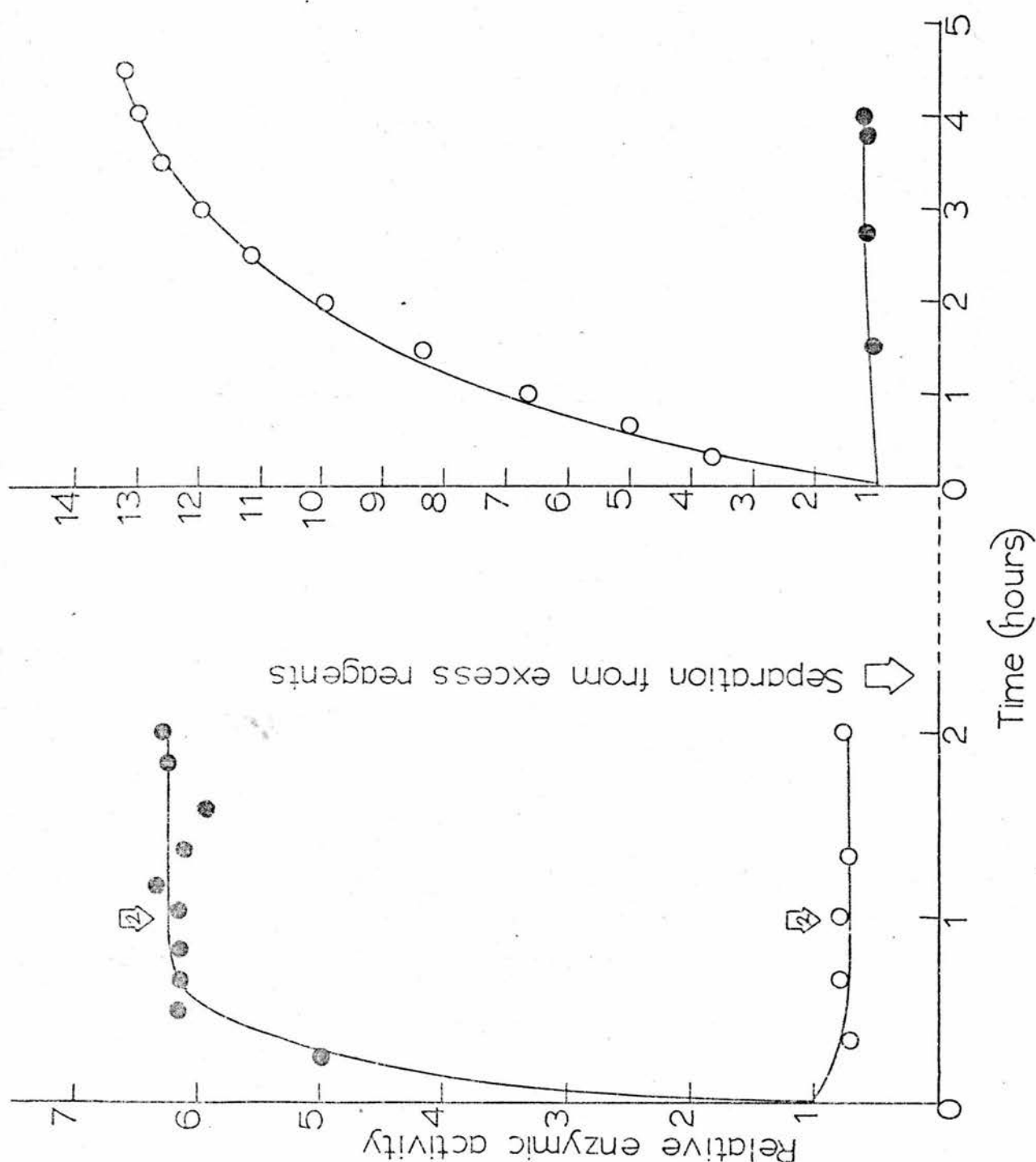


Fig. 6-5. The reaction of MPI with LADH previously modified with ethyl acetimidate. Stage-1: Acetimidation: buffer, 0.5M N-ethylmorpholine HCl, pH 8.0; 23.5°C; •, without added ligand; enzyme, 28.5 μ M; o, DPNH (170 μ M) plus isobutyramide (55mM), enzyme, 53.8 μ M. Two additions of ethyl acetimidate (0.1M) were made, the first at time zero and the second at a time indicated by ②. Stage-2: Picolinimidylation after removal of reagents by column filtration: the acetimidated enzyme was treated with 0.1M MPI in 0.5M N-ethylmorpholine HCl, pH 8.0 at 23.5°C.

phosphate plus 0.25M chloride gave a 9-fold activation indicating that varying ionic strength and chloride binding have an insignificant effect on activation. Phosphate seems to have a specific effect, since, (a) ionic strength is unimportant, (b) chloride has no effect, (c) replacing chloride by phosphate as the anion in N-ethylmorpholine buffer reduces activation by half and, (d) increasing phosphate concentration results in decreasing activation. A possible effect is that the picolinimidated enzyme is less stable in the presence of phosphate and MPl.

Estimation of the number of lysine residues protected by DPNH and AMP

The enzyme was initially acetimidated in the presence of excess protecting ligand in 0.5M N-ethylmorpholine-HCl, pH 8.0, at 23.5°C, for 2 hours. Solid ethylacetimidate, sufficient to give a concentration of 0.1M, was added at zero time and after one hour. The acetimidated enzyme was then separated from excess reagent by passing through a column of sephadex-G25 (medium) (0.9 x 40cm) equilibrated with 0.5M N-ethylmorpholine-HCl, pH 8.0 at room temperature. The solution was then treated with MPl for 4 hours and MPl removed by passing through a column of sephadex-G25 (fine) (0.9 x 30cm) equilibrated with 50mM phosphate, pH 6 at room temperature. It was found that MPl was difficult to remove from the column by elution with distilled water or buffer and washing with N NaOH was necessary to elute the reagent. Absorbance at 260nm was used as the criterion for the presence of MPl in the effluent. The number of picolinimidated lysine residues in the modified enzyme and

the protein concentration were determined.

In the ternary complex of LADH with DPNH and isobutyramide 4.5 lysines were protected per subunit compared to three residues found by Plapp (1970). However, 4.5 picolinimidyl groups were also calculated to be incorporated into enzyme which had been acetimidated in the absence of protecting ligand. Although, there was negligible additional activation, DPNH and AMP protection resulted in similar incorporation of picolinimidyl groups. It appears that there was considerable incorporation of picolinimidyl groups at lysine residues which had perhaps been exposed by denaturation of the protein after acetimidation and during picolinimidation. No conclusions can be given, therefore, about the number of lysines directly protected by ligands.

Binding of AMP to picolinimidated LADH

The dissociation constant of AMP with picolinimidated LADH was determined kinetically at pH 10 (10m glycine-NaOH and 8.25mM ethanol; DPN^+ 4mM-17mM) by competition with DPN^+ . The kinetics were performed fluorimetrically as described previously. The dissociation constant was determined to be $3,800 \mu\text{M}$, which is an 8-fold increase on the dissociation constant of AMP and native enzyme, measured to be $460 \mu\text{M}$ at pH 10. Plapp (1970) found that AMP binding at pH 9.0 was unchanged in picolinimidated LADH compared to native enzyme.

It was also attempted to measure the binding of AMP at pH 7 and pH 8. However, the rate of DPN^+ reduction or DPNH oxidation at high substrate concentrations decreased

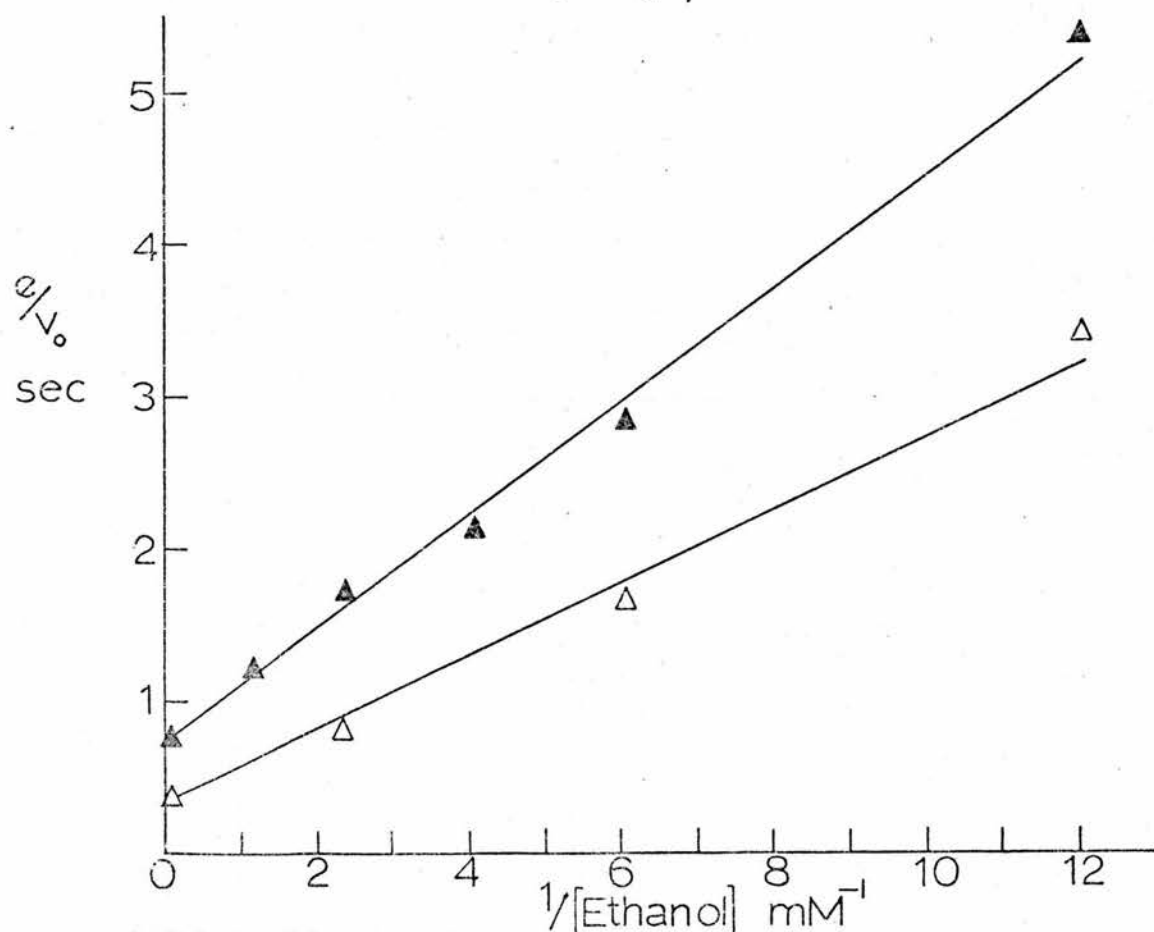
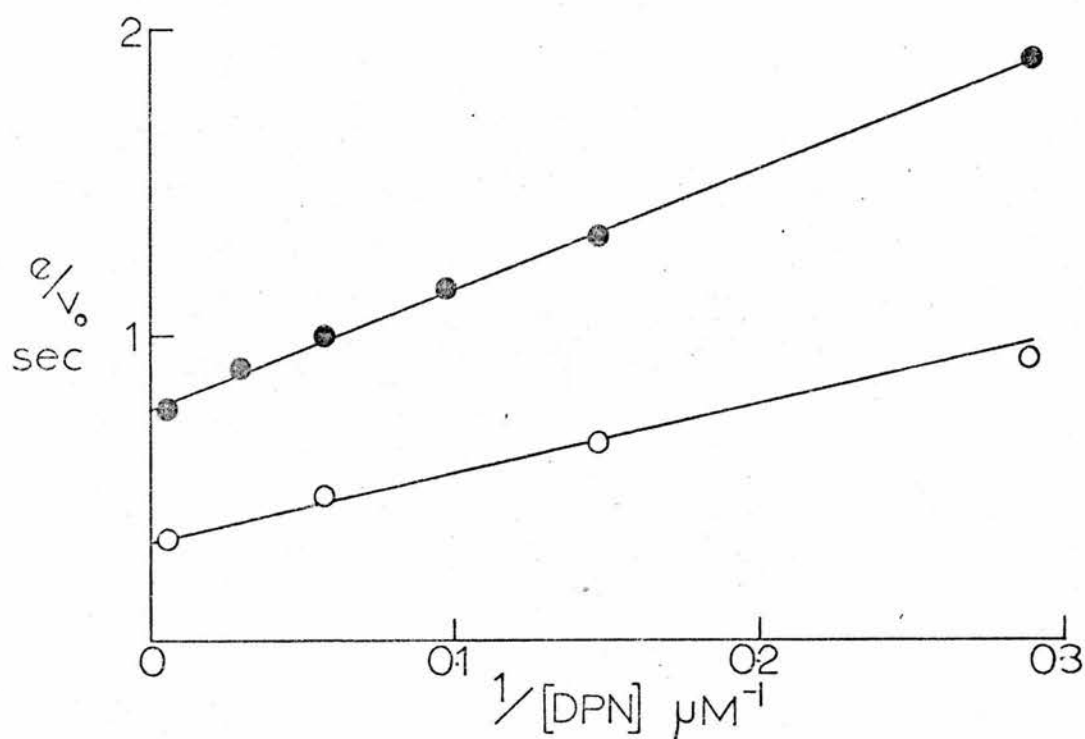


Fig.6-6. Kinetics in the presence of MPI at constant ethanol concentration (8.25mM) and varied DPN^+ concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; o, without MPI; ●, + MPI (2.4mM).

Fig.6-7. Kinetics in the presence of MPI at constant DPN^+ concentration (250 μM) and varied ethanol concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; Δ, without MPI; ▲, + MPI (2.0mM).

too rapidly after addition of enzyme to allow measurement of the initial rate. This burst of activity could be repeated by subsequent additions of picolinimidated enzyme. This effect did not appear to be due to rapid inactivation of the enzyme since addition of ethanol to start the reaction after incubation of enzyme in the assay cuvette produced the same result. Moreover, the picolinimidated enzyme was relatively stable for one hour at 23.5°C over the pH range 6-10 when diluted to 0.034mg/ml.

Binding of MPL to native LADH

The binding of MPL to LADH at pH 7 was investigated kinetically. The time required to obtain an accurate initial velocity after addition of enzyme is short enough (20 secs.) to ignore effects due to modification of the enzyme. MPL inhibited LADH, double reciprocal plots showing uncompetitive non-competitive inhibition with respect to both DPN^+ and ethanol as shown in fig. 6-6. The lines in both cases intersect below the abscissa. At pH 7 MPL is a cation and auramine O, another cationic compound, exhibits non-competitive inhibition of LADH with respect to both ethanol and DPN^+ at pH 7.4 (Conrad et al., 1970). Since MPL has a structural similarity to auramine O it is possible they are inhibiting by similar mechanisms and binding at sites distinct from the active sites. This inhibition of LADH by MPL may be a clue to the course of effects observed during attempts to do kinetics with picolinimidated enzyme at pH 7 and pH 8. It is possible that there is a time dependent inhibition of picolinimidated LADH by a bound cationic picolinimidyl group.

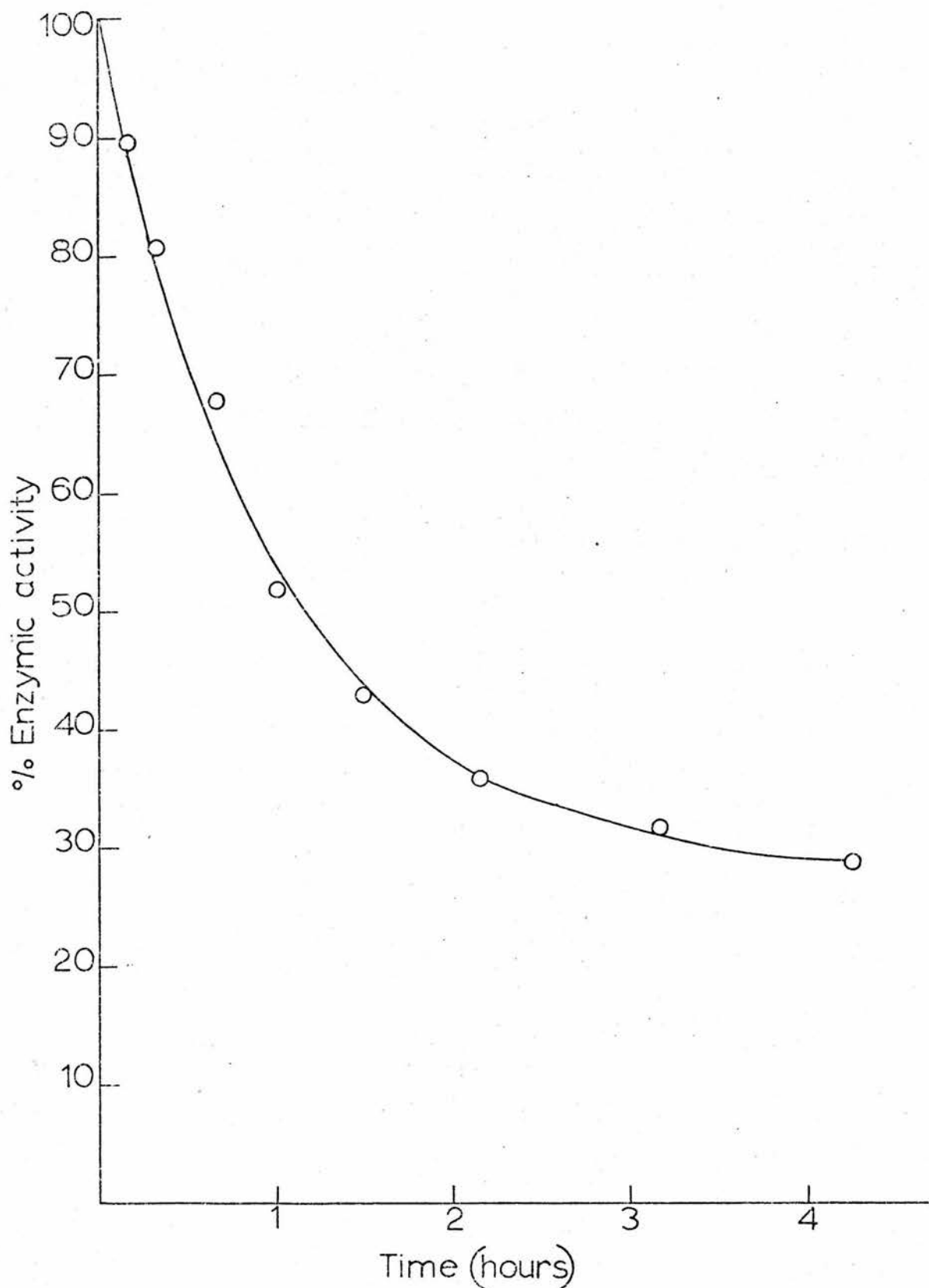


Fig. 6-8. Reaction of MPI with carboxymethylated-LADH. CM-LADH, $14\ \mu\text{M}$; 0.1M MPI; 0.5M N-ethylmorpholine HCl, pH 8.0; 23.5°C . Assay conditions: 0.1M glycine-NaOH, pH 10.0, 1.80mM DPN^+ , 0.5M ethanol. Activity was related to the enzyme activity of the incubation solution before addition of MPI at time zero.

Picolinimidation of carboxymethyl-LADH

The effect of reaction of CM-LADH with MP1 is shown in fig. 6-7. There is no indication of any enhancement of activity, but inactivation to a residual activity of 30% occurs after 4 hours. This residual activity may be due to a small amount of native enzyme present in the CM-LADH. Assuming a 12-fold activation of this native enzyme less than 3% would account for the residual activity.

Inactivation may be due to unfolding of the CM-LADH structure, which is less stable than native enzyme, otherwise it may be an indication of different enzyme kinetics for CM-LADH. Reynolds and McKinley-McKee (1970) have suggested that the rate of breakdown of the enzyme-coenzyme complexes is not rate determining in CM-LADH activity.

Picolinimidation of PLP-LADH

This experiment, designed to provide additional proof that the residual activity of PLP-LADH is due to a proportion of the modified enzyme with the active centre lysine unmodified, is described in table 6-1.

Activation and inactivation occurred concomitantly with inactivation predominating. However, after 138 minutes the percentage activity measured by assay (2) was 10-fold greater than the activity measured by assay (1). It is probable that activation of LADH by MP1 is due to modification of the same active centre lysine which PLP reacts with to inactivate the enzyme. The above result, therefore, strongly suggests that the residual activity of PLP-LADH is due to PLP-LADH with the active centre lysine unmodified.

TABLE 6-1The reaction of MPL with reduced PLP-LADH

PLP-LADH ($20.1\mu\text{M}$), with 9.2 PLP residues incorporated per subunit and with a residual enzymic activity of 14%, was incubated with 0.16M MPL in 0.5M N-ethylmorpholine-HCl, pH 8.5. Activity measurements were made using the following assays.

- (1) 0.42mM DPN⁺, 8.25mM ethanol and 62mM glycine-NaOH, pH 10.
- (2) 2.1mM DPN⁺, 550mM ethanol and 62mM glycine-NaOH, pH 10.

<u>Time (mins.)</u>	<u>% Activity (Assay (2))</u>	<u>% Activity (Assay (1))</u>
0	100	100
15	134	
31	162	
46	176	
61	170	
91	137	
95 (a second addition of MPL was made giving a total concentration of 0.32M)		
114	109	
138	91	9

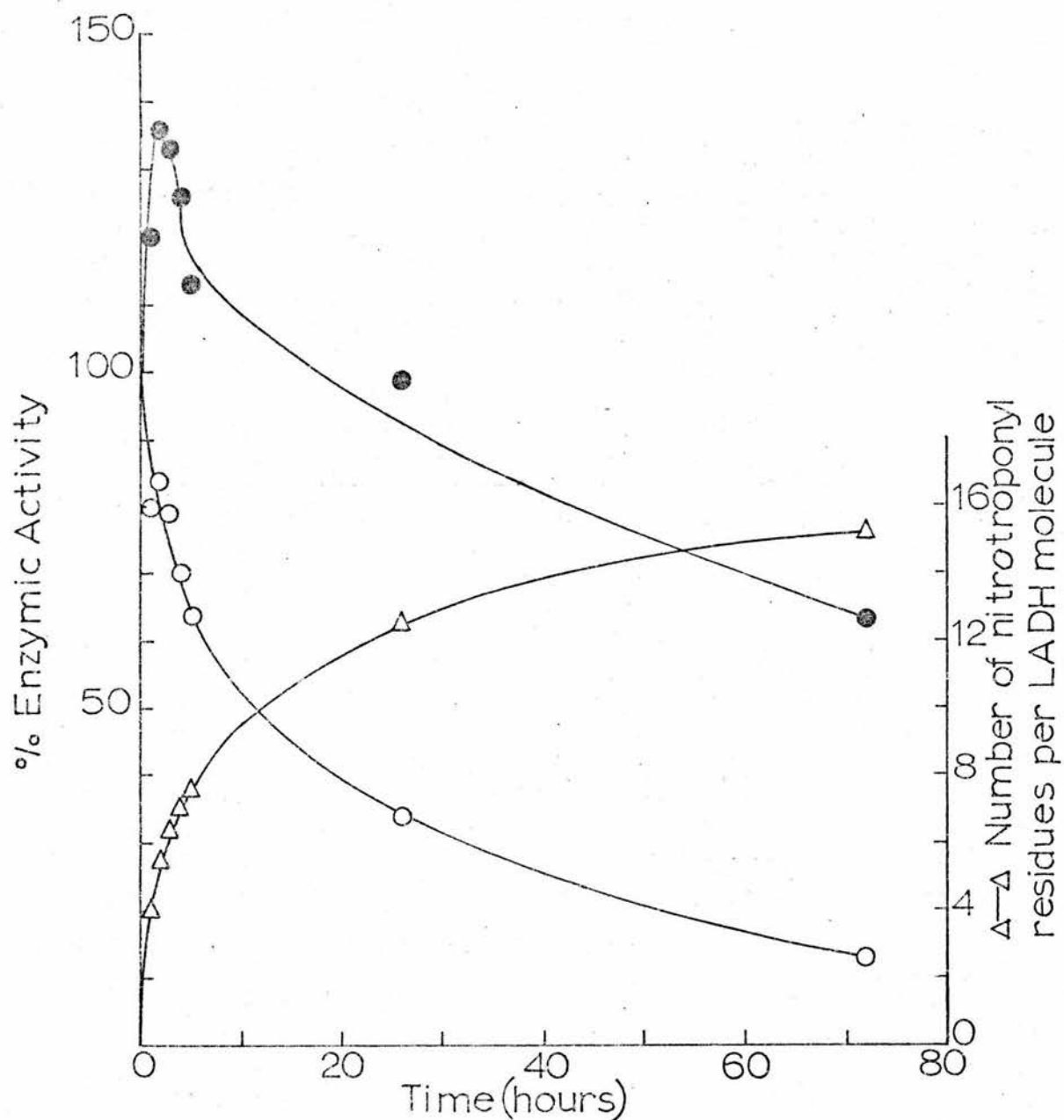


Fig.6-9. The effect of 2-Methoxy-5-Nitrotropone (MNT) on LADH. 50 M LADH was incubated at room temperature in 0.5M NEthylmorpholine-HCl, pH 8.5, containing 10mM MNT. Activity was assayed in; (1) 0.42mM DPN⁺, 8.25mM ethanol and 62mM glycine-NaOH, pH 10, (O); (2) 2.1mM DPN⁺, 550mM ethanol and 62mM glycine-NaOH, pH 10, (●).

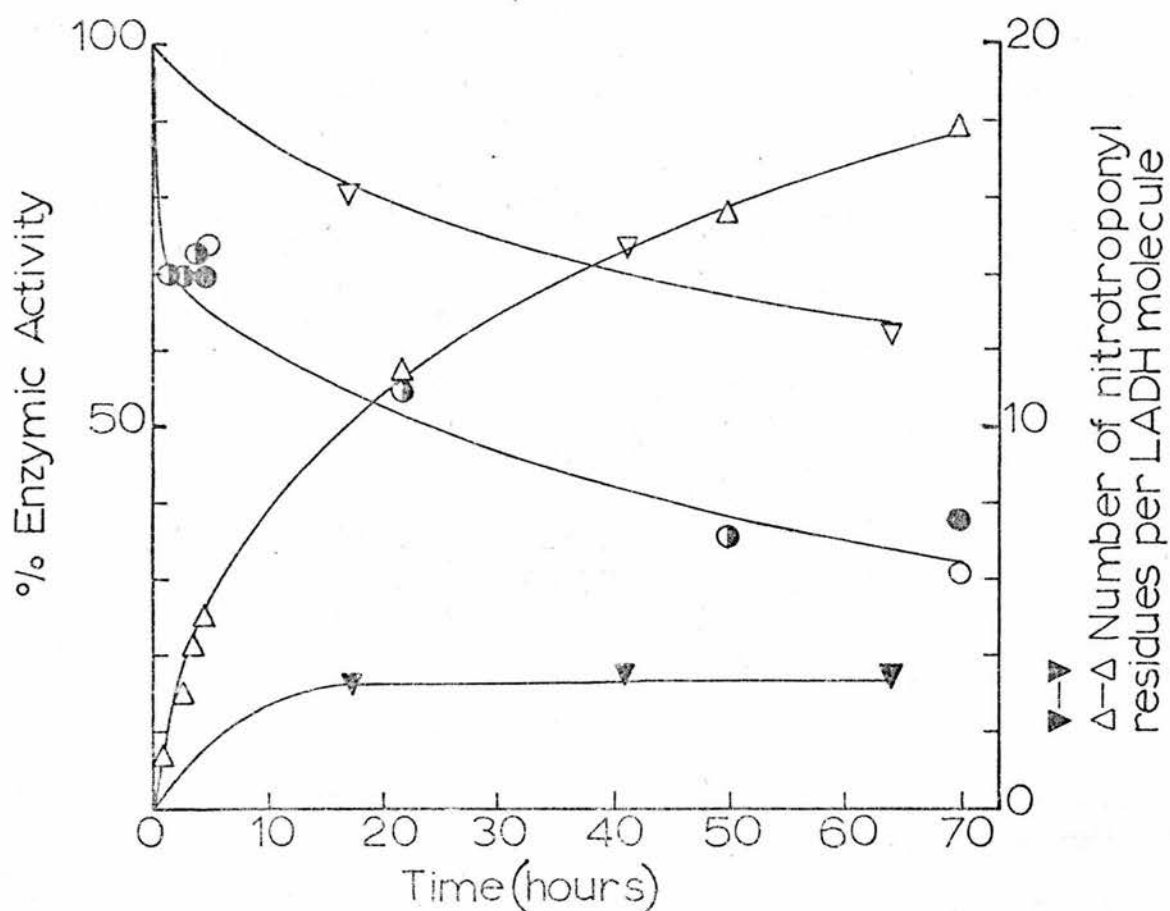


Fig.6-10. The effect of AMP and DPNH on the inactivation of LADH by 2-Methoxy-5-Nitropropone.

The following assay solutions were used: (1) 0.42mM DPN⁺, 8.25mM ethanol and 62mM glycine-NaOH, pH 10. (2) 2.1mM DPN⁺, 550mM ethanol and 62mM glycine-NaOH, pH 10.

LADH was incubated at room temperature in 0.5M N-ethylmorpholine-HCl, pH 8.5, and 10mM MNT under the following conditions:

(a) LADH, 51 μ M; AMP, 137mM; o, activity measured by assay (1); ●, activity measured by assay (2); ●, points at which the percentage residual activity measured by both assays (1) and (2) was the same; Δ, the number of nitropropionyl residues incorporated per LADH molecule.

(b) LADH, 54.7 μ M; DPNH, 3.25mM; ▽, points at which the percentage residual activity measured by both assays (1) and (2) was the same; ▽, the number of nitropropionyl residues incorporated per LADH molecule.

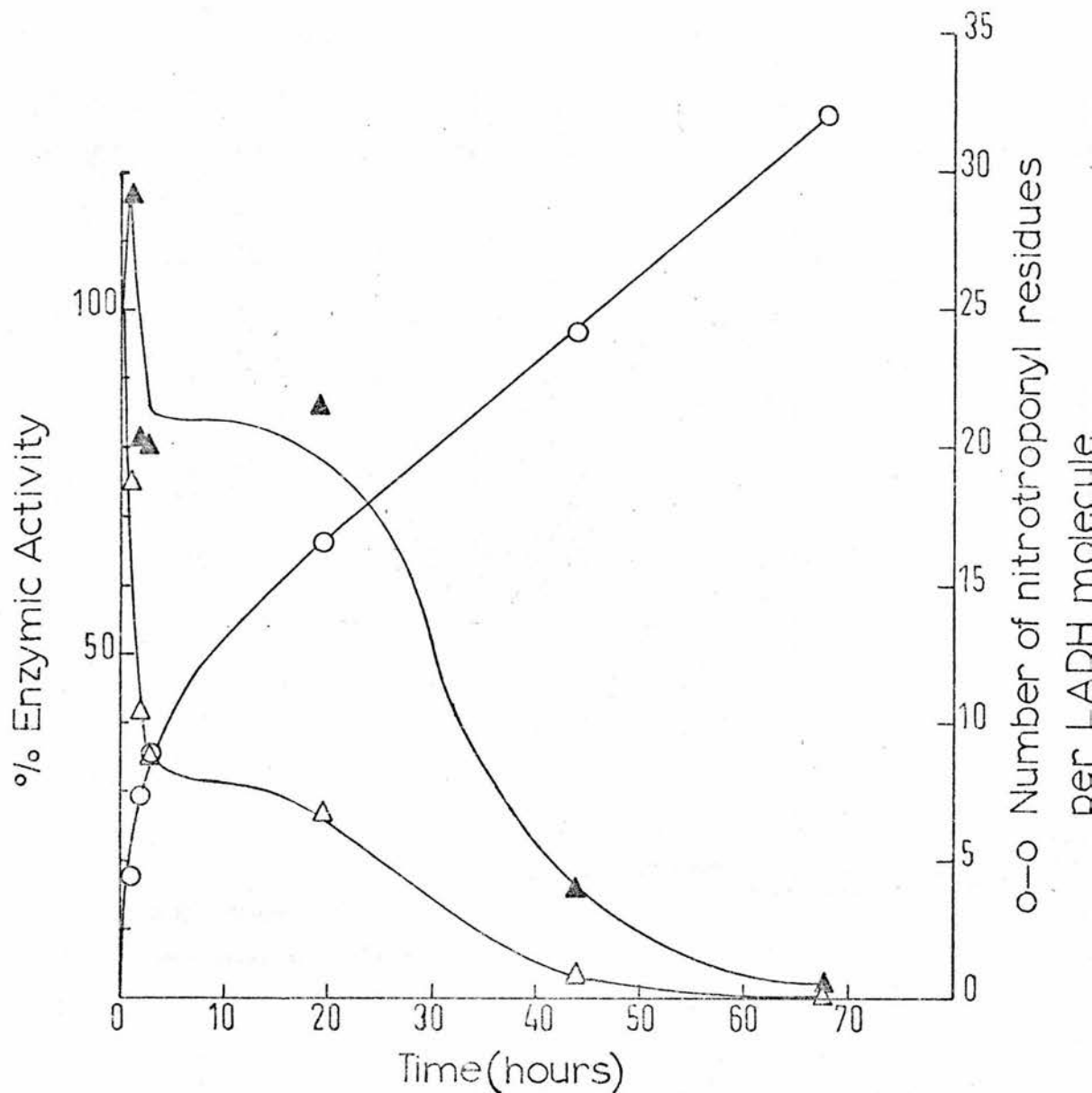


Fig.6-11. The effect of Imidazole on the inactivation of LADH by 2-Methoxy-5-Nitrotropone. $54.7 \mu\text{M}$ LADH was incubated at room temperature in 0.5M N-ethylmorpholine-HCl, pH 8.5, containing 10mM MNT and 78mM imidazole. Activity was assayed in; (1) 0.42mM DPN^+ , 8.25mM ethanol and 62mM glycine-NaOH, pH 10, (Δ); (2) 2.1mM DPN^+ , 550mM ethanol and 62mM glycine-NaOH, pH 10, (\blacktriangle).

Reaction with MNT

Reaction of the lysines of LADH with MNT proved to be very much slower than reaction with PLP or MPl. Fig. 6-9 shows that activation and inactivation are occurring concomitantly. The activation is abolished by the binding of AMP or DPNH to the enzyme (fig. 6-10). AMP and DPNH also protect against inactivation, DPNH more effectively than AMP. This is probably because DPNH markedly reduces the number of nitrotroponyl residues incorporated, whereas AMP has little effect. Imidazole produces a considerable increase in both the rate and the extent of incorporation of nitrotroponyl residues into the enzyme (fig. 6-11). After an initial rapid phase the curve in fig. 6-11 is linear and 32 nitrotroponyl residues per LADH molecule were incorporated after 68 hours. These effects were accompanied by an increased rate of activation and inactivation, such that after 68 hours less than 2% of initial activity remained when measured by either assay (1) or (2) as illustrated in fig. 6-11.

Inactivation of LADH by MNT was partially reversed by removal of the incorporated nitrotroponyl groups. After 72 hours incubation the solution used in the experiment illustrated by fig. 6-9 was made 1.0M in hydrazine, the pH was adjusted to 9.0 and incubation continued for 1 hour. This treatment reduced the number of nitrotroponyl residues incorporated from 15.3 to 1.7 per LADH molecule. The activity measured by the Dalziel assay (assay (1) in table 6-1) increased from 13% to 27% whereas the activity measured by assay (2) decreased from 63% to 45%.

Discussion

The results obtained from modification of the lysines of LADH with MPl are consistent with the postulated existence of one lysine residue per coenzyme binding site, situated so as to attract the phosphate of the AMP moiety. DPNH protected completely and AMP protected very strongly, the partial activation perhaps being due to AMP not completely covering the essential lysine thus leaving limited access to MPl.

The suggestion that there is a lysine at the nicotinamide binding site (Plapp, 1970) is unlikely since AMP, being an anion, would not be expected to protect as strongly as is observed against the picolinimidation of such a residue. Moreover, decanoate and imidazole, which bind to the active centre zinc do not protect against activation. Indeed, imidazole actually accelerates activation. The lack of protection by decanoate supports the suggestion that competition between decanoate and PLP is due to electrostatic repulsion between the phosphate group of PLP and the fatty acid carboxylate group and that steric interference does not play any part. However, orthophenanthroline and bipyridyl protect appreciably.

These zinc chelating ligands may protect partially by producing a conformational change on binding. Since excess of ligand was present, the binding site at which they produce the change need not be the active centre zinc atom. The increased rate of activation by imidazole may be also due to a conformational change increasing the exposure of the essential lysine to attack by MPl. It is

unlikely that orthophenanthroline and 2,2'-bipyridyl protect by steric interference with reaction of MPI with a lysine at the AMP binding site since orthophenanthroline and AMP bind non-competitively (Yonetani and Theorell, 1964). Plapp (1970) moreover, showed that binding of 2,2'-bipyridyl was unchanged by picolinimidation. The effect of imidazole and orthophenanthroline on inactivation of LADH by PLP indicated that conformational changes might be involved (Chapter 5) and it has also been shown that imidazole accelerates the rate of reaction of iodoacetate with the iodoacetate reactive thiol of LADH (Evans and Rabin, 1968), which has also been suggested to be due to a conformational change produced by imidazole binding.

BPCA, which was shown to be strictly competitive with PLP and does not bind to the active centre zinc (Sigman, 1967), protected partially at saturating concentrations. Although BPCA is probably bound by the carboxylate group to the ϵ -ammonium group of the active centre lysine it does not provide as much protection as the phosphate of AMP. The rest of the AMP molecule may play an important role in protecting the lysine perhaps by inducing a conformational change in the enzyme.

At pH 10 it was shown that the binding of AMP to the picolinimidated enzyme is 8-fold weaker than binding to native LADH. At pH 9.0 Plapp (1970) found, comparing picolinimidated enzyme with native enzyme, that AMP binding was unchanged, whereas DPN^+ binding was weakened 53-fold and DPNH binding weakened 12-fold. Modification of the ϵ -amino group of the essential lysine with MPI preserves

the positive charge but introduces a group which apparently interferes sterically with the binding of AMP. Coenzyme binding would be expected to be weakened even more seriously since in order for the nicotinamide moiety to bind near the active centre zinc atom, the coenzyme molecule can be envisaged as having to surmount the obstacle of the picolinimidyl group.

Plapp did not observe a weakening of AMP binding to picolinimidated LADH at pH 9.0 compared with native LADH. However, the pK of the picolinimidated lysine group would be significantly higher than the pK of the ϵ -amino group of lysine (compare the pKs of the guanidine group of arginine and the ϵ -amino group of lysine). The dissociation constant measured at pH 9.0 by Plapp may be close to the acid asymptote value for the variation of the dissociation constant of AMP and picolinimidated LADH with pH. This would mean about a 6-fold weakening of AMP binding, comparing a $K_{E,AMP}$ of $415 \mu M$ for picolinimidated LADH at pH 9.0 with a $K_{E,AMP}$ of $69 \mu M$ for native LADH at pH 7.1.

The picolinimide of one lysine per subunit of LADH situated at the AMP binding site of the active centre and serving to attract the phosphate of AMP and the corresponding phosphate of the coenzyme seems to be sufficient to explain the protection effects of inhibitors and co-enzymes against activation of LADH by MP1 and the kinetic and nucleotide binding properties of the modified enzyme. Modification of lysine residues in LADH with MNT results in eventual inactivation which is only partially reversed by removal of the nitropropionyl residues. This suggests

that denaturation of the protein occurs caused by the disruption of the charge balance when the ϵ -ammonium groups of lysine residues are replaced by neutral nitro-troponyl groups.

Activation also occurs concomitantly with inactivation and since this is prevented by protection with AMP or DPNH this is probably due to modification of the postulated active centre lysine, which binds the phosphate of AMP. In this case, the activation is due to weakening of coenzyme binding and is very similar to the effect produced by MPl. The weakening of coenzyme binding by the nitro-troponyl residue is due to two effects;

(1) Steric interference with coenzyme binding; (2) abolition of the positive charge of the active centre lysine. These results, therefore, suggest that the positive charge of the active centre lysine, although contributing to the binding of the coenzyme, is not essential for coenzyme binding.

Further evidence is also obtained for a conformational change, produced by imidazole, which exposes the lysine residues so they react more readily with MNT or MPl. On the other hand the conformation of the enzyme DPNH complex appears to be much more compact, so that the lysine residues are protected from MNT.

CHAPTER 7.

Kinetic Studies on the Binding of Iodoacetate and other
Anions to Liver Alcohol Dehydrogenase

Introduction

It has been shown that iodoacetate forms a reversible complex with LADH as well as inactivating the enzyme by carboxymethylating cysteine-46 in each subunit. Inactivation has been shown to follow Michaelis-Menten-type kinetics and this has been used to investigate the binding of iodoacetate and known inhibitors as well as interactions between the inhibitors (Reynolds and McKinley-McKee, 1969; Reynolds et al., 1970). It was found that iodo-carboxylic acids and aromatic carboxylic acids bound to the enzyme in a similar manner. By using steady state kinetics it has been possible to study interactions between inhibitors as well as the binding of iodoacetate. 4-Biphenyl carboxylic acid and phenylacetic acid were used in these kinetic studies as inert analogues of iodoacetate.

Results

Iodoacetate

Attempts by Reynolds (1970) to measure the formation of the reversible complex of LADH with iodoacetate failed because a rapid time dependent loss of DPNH fluorescence occurred in the presence of iodoacetate. However, incubation of low concentrations of DPNH ($0.2 \mu\text{M}$) and high concentrations of DPNH ($23 \mu\text{M}$) with iodoacetate (6.25mM) in phosphate buffer, pH 7.14 at 23.5°C in the fluorimeter described in Chapter 2 resulted in no loss of DPNH fluorescence over time periods required for initial rate determinations. The interaction of DPNH with iodoacetate may be a photochemical reaction requiring a greater light intensity than that supplied by the mercury lamp. Also,

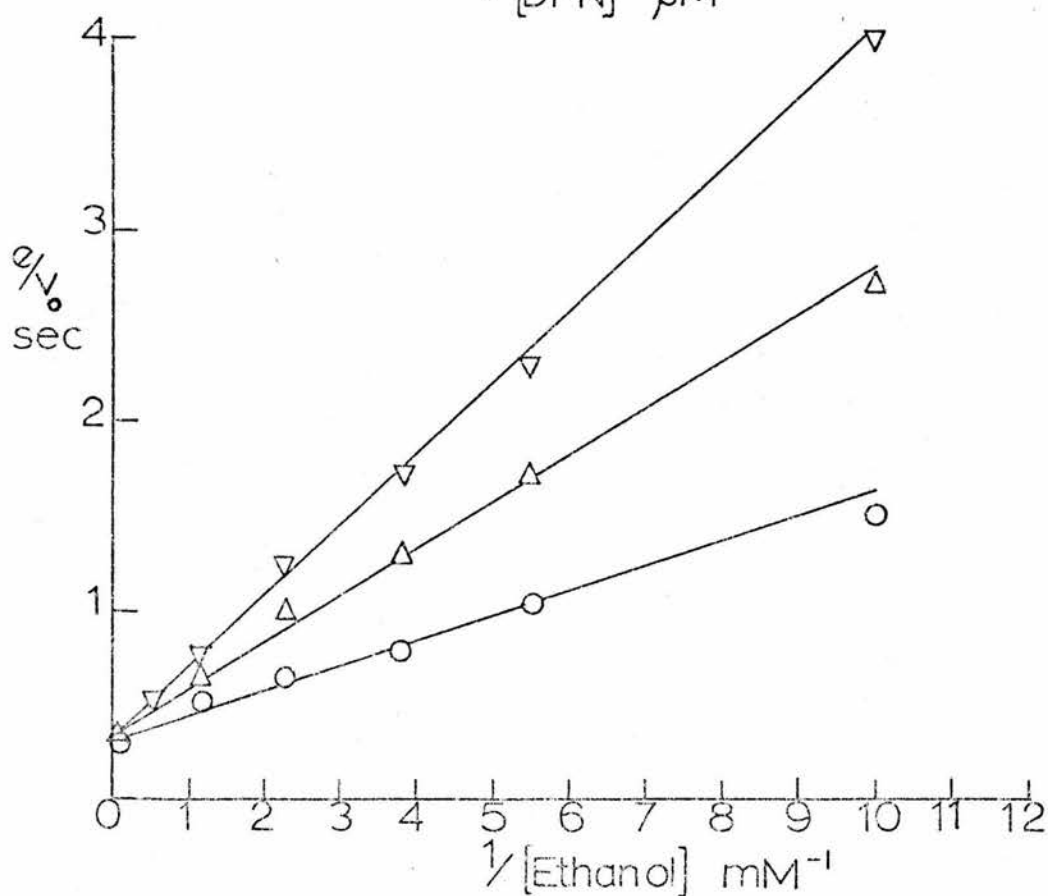
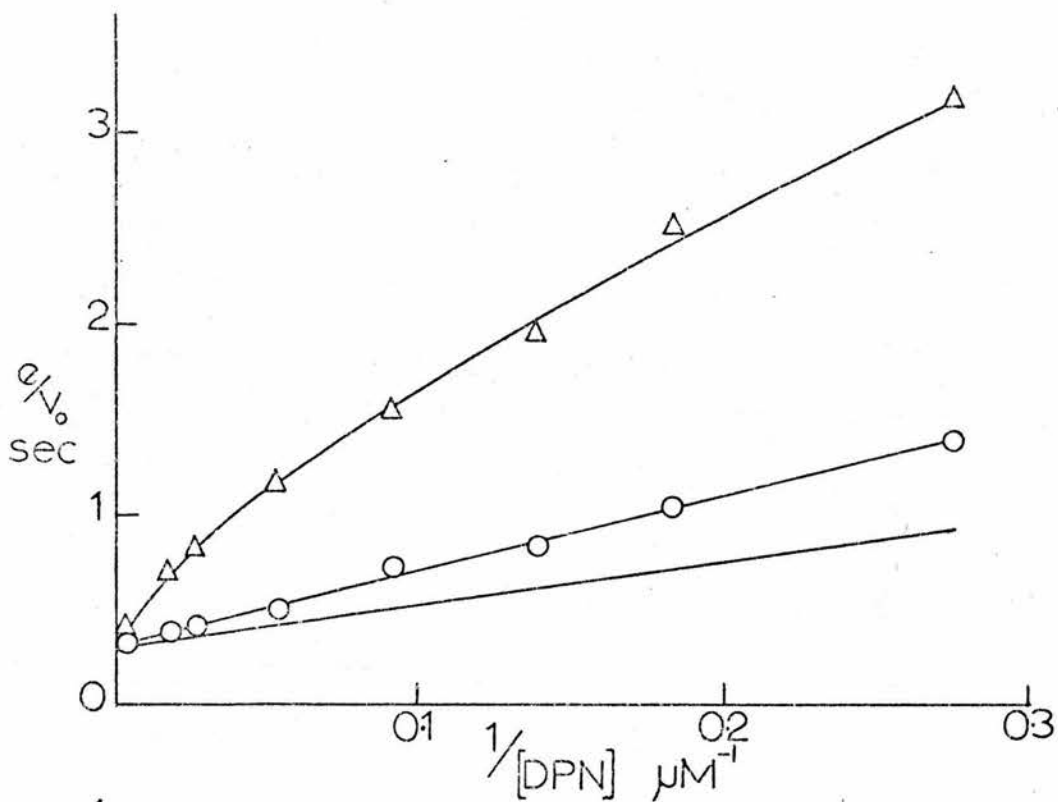


Fig.7-1. Kinetics in the presence of iodoacetate at constant ethanol concentration (8.25mM) and varied DPN^+ concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C ; — without iodoacetate; + iodoacetate, O, (10.75mM), Δ, (21.50mM).
 Fig.7-2. Kinetics in the presence of iodoacetate at constant DPN^+ concentration (250 μM) and varied ethanol concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C ; O, without iodoacetate; + iodoacetate, Δ, (1.34mM), ▽, (2.69mM).

reaction cuvettes containing phosphate buffer, pH 7.14, ethanol (8.25mM) DPN⁺ (250 μ M), and iodoacetate (10.57mM), which had been incubated at 23.5°C for 40 minutes gave the same initial rate as a similar cuvette which was measured immediately. The same result was found when 6 μ M DPN⁺ was used. It thus seemed feasible to measure initial rates in the presence of iodoacetate. Abeles and Lee (1960) had previously shown that iodoacetate inhibits the aldehyde mutase reaction competitively with formaldehyde ($K_I = 5\text{mM}$).

Fig. 7-2 shows that iodoacetate is strictly competitive with ethanol with a K_I calculated from the slopes of 1.36mM.

Iodoacetate is competitive with DPN⁺ (fig. 7-1). When high concentrations of iodoacetate (21.5mM) were present the Lineweaver-Burke plot is concave-down. When the iodoacetate concentration was 10.73mM the plot was straight and K_I was 14.2mM. This is several times larger than the dissociation constant of 4.5mM found by Reynolds and McKinley-McKee, (1969). However, when the slope of the tangent at the intercept of the curved plot with the vertical axis was used K_I was calculated to be 2.3mM.

The measurement of the initial rate of reduction of acetaldehyde by DPNH in the presence of iodoacetate proved difficult because of a rapid decrease in initial rate. However, the results obtained with varying acetaldehyde concentration at constant DPNH (13 μ M) concentration in the presence of iodoacetate (6.72mM) indicate that inhibition is probably uncompetitive.

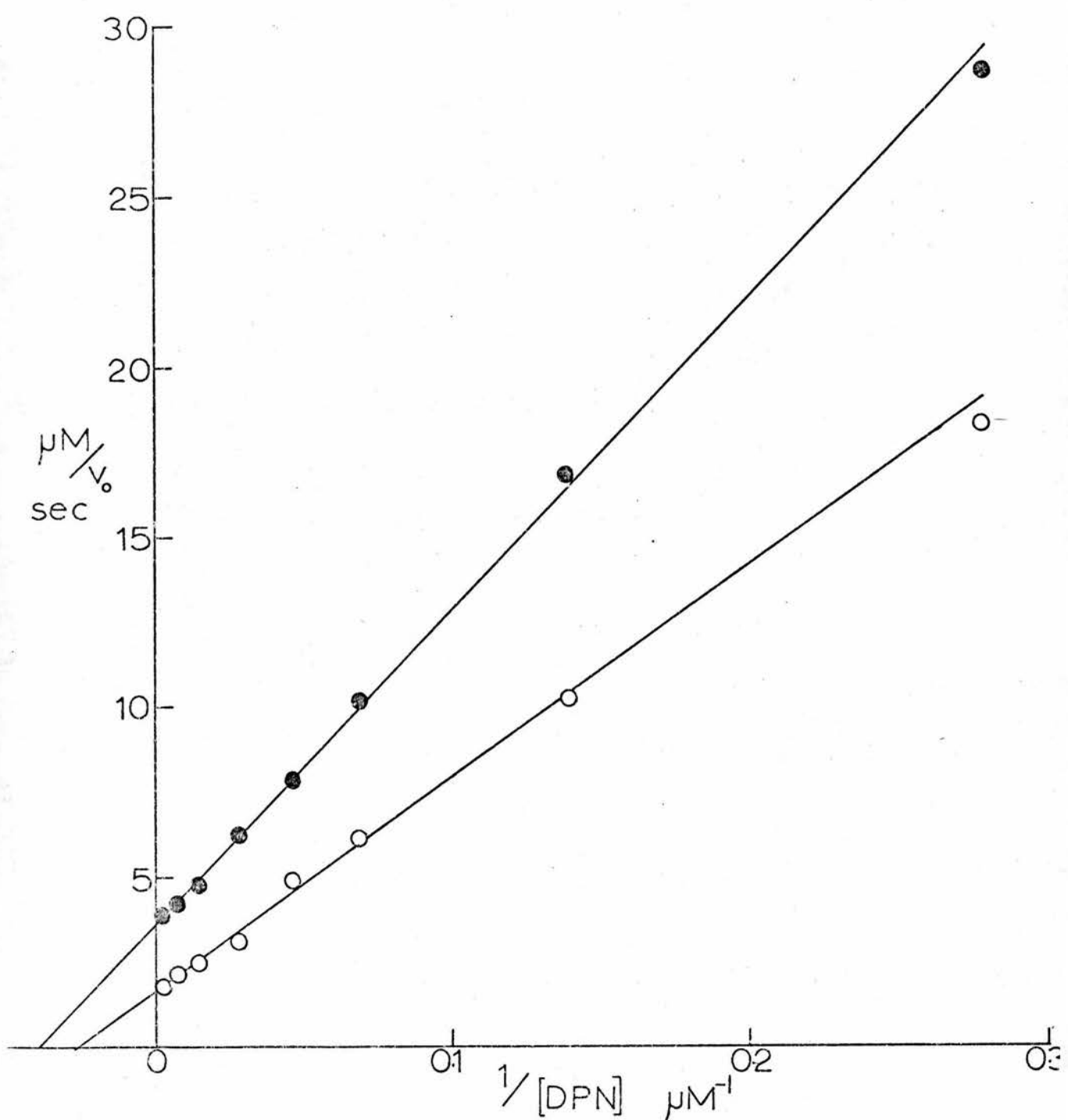


Fig.7-3. The effect of iodoacetate on DPN^+ - reduction by butanol (5.47mM) and carboxymethylated-LADH. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C ; o, without iodoacetate; ●, + iodoacetate (6.72mM).

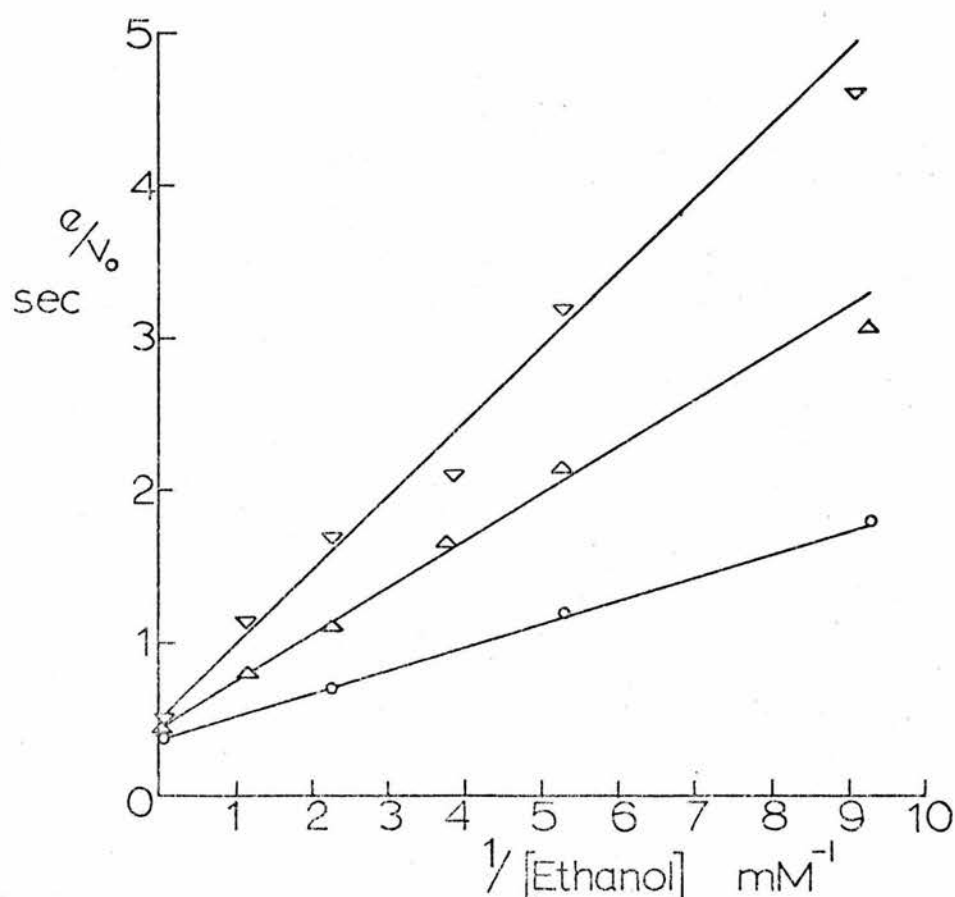
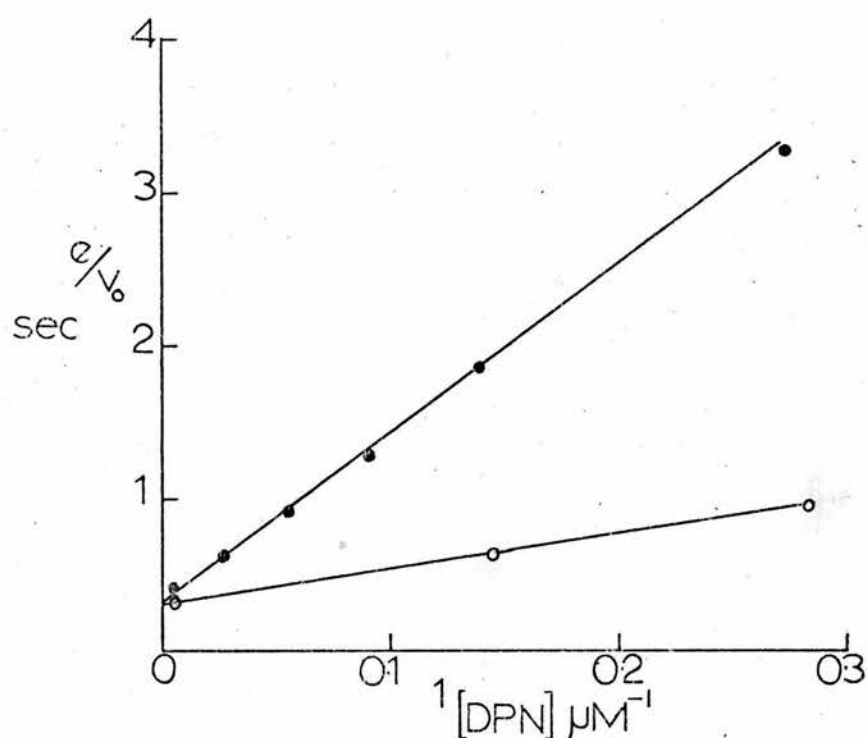


Fig. 7-4. Kinetics in the presence of BPCA at constant ethanol concentration (8.25mM) and varied DPN^+ concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; o, without BPCA; ●, + BPCA (126 μM).

Fig. 7-5. Kinetics in the presence of BPCA at constant DPN^+ concentration (484 μM) and varied ethanol concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; o, without BPCA; + BPCA, Δ, (126 μM), ▽, (252 μM).

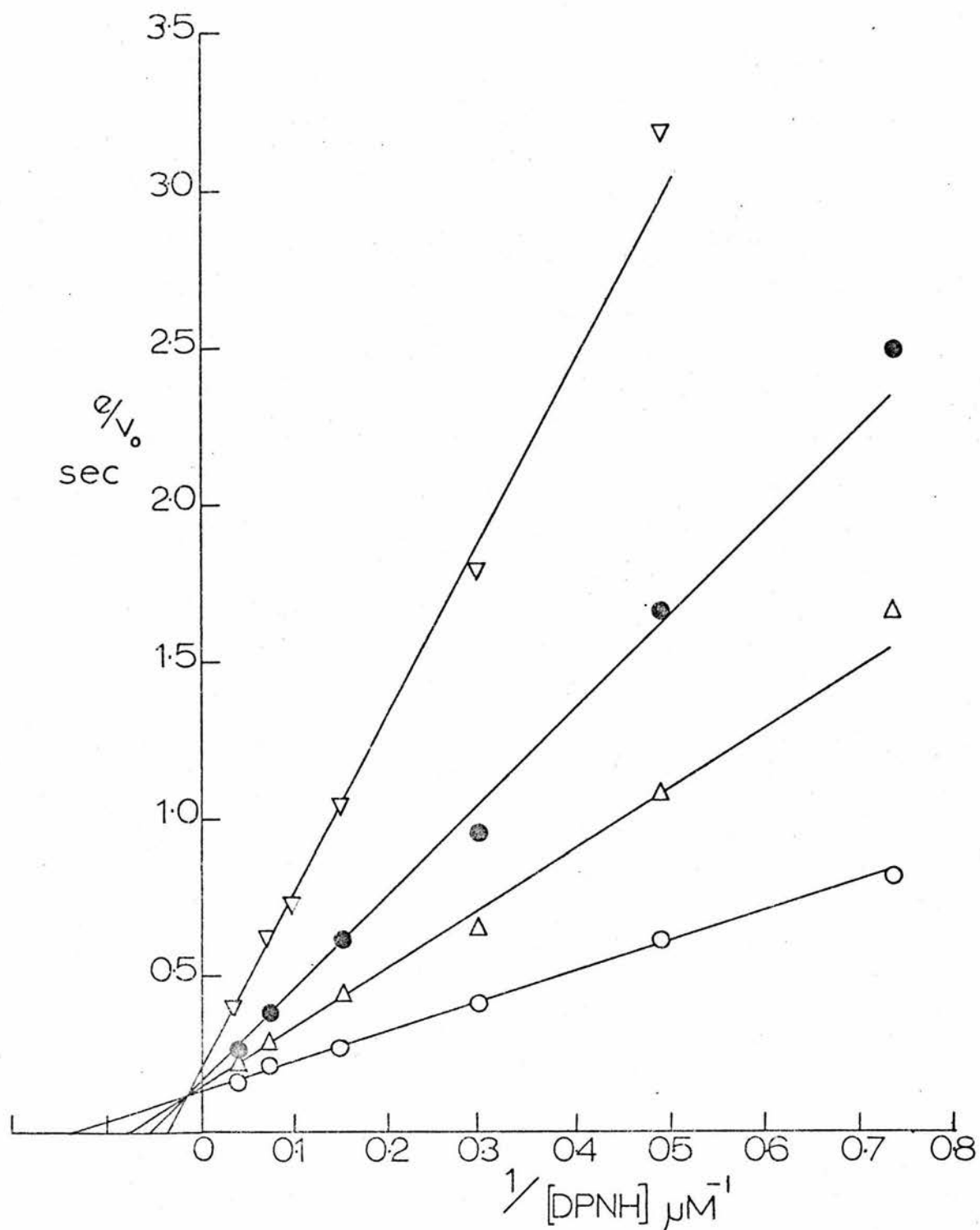


Fig.7-6. Kinetics in the presence of BPCA at constant acetaldehyde concentration (3.70mM) and varied DPNH concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; o, without BPCA; + BPCA, Δ , (31.5 μM), \bullet , (63.0 μM), ∇ , (126.0 μM).

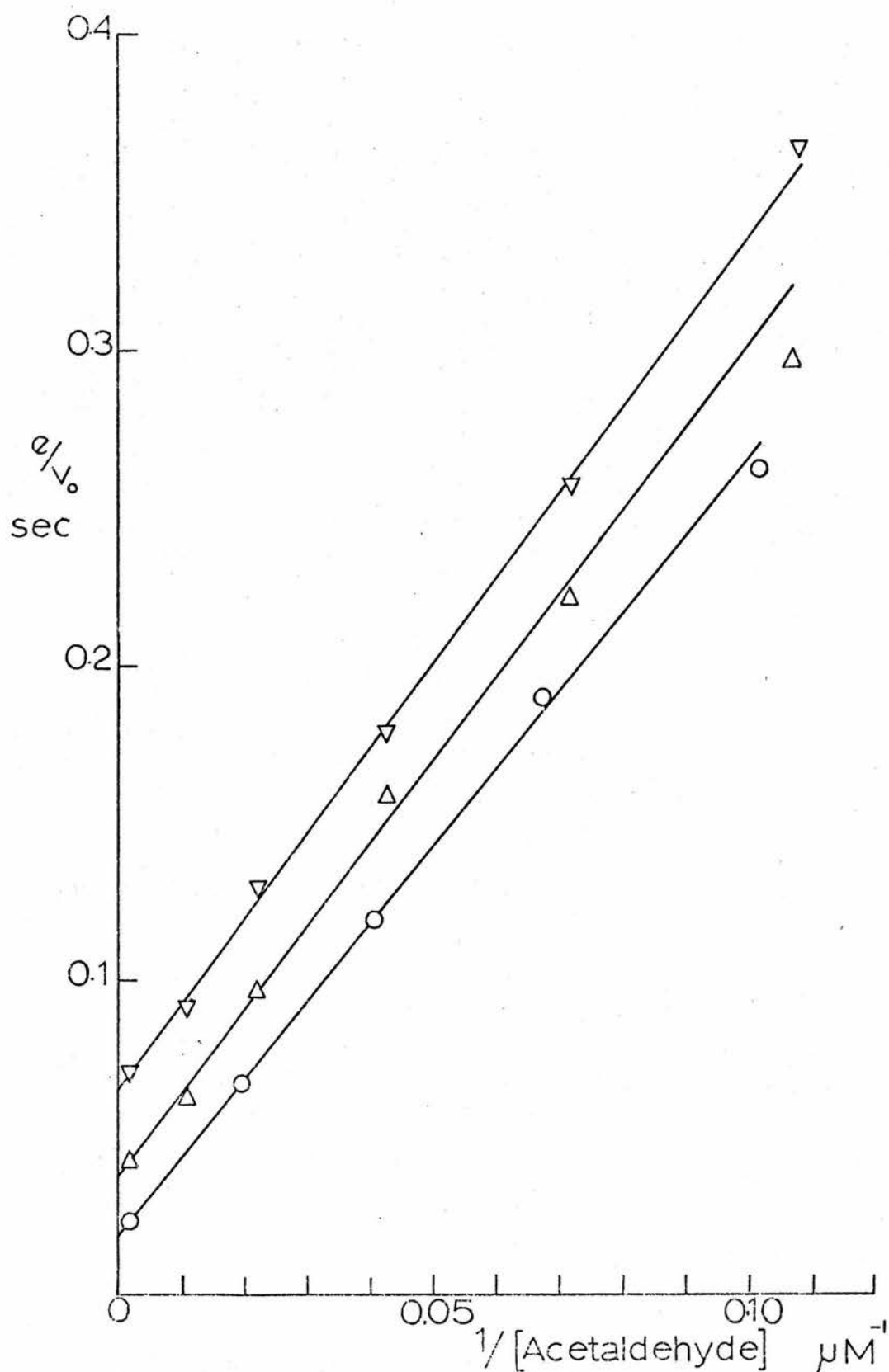


Fig.7-7. Kinetics in the presence of BPCA at constant DPNH concentration ($13.7 \mu\text{M}$) and varied acetaldehyde concentration. Phosphate buffer, pH 7.14, ionic strength 0.1; o, without BPCA; + BPCA, Δ , ($63 \mu\text{M}$), ∇ , ($126 \mu\text{M}$).

The inhibition of CM-LADH by iodoacetate was investigated and found to give mixed inhibition when DPN^+ is varied (fig. 7-3). Formation of a ternary iodoacetate complex is indicated as well as competition of iodoacetate with DPN^+ . A value of 13.7mM was calculated for K_I from the slopes.

4-Biphenylcarboxylic acid

BPCA is strictly competitive with DPN^+ as shown in fig. 7-4. A value of $32\mu\text{M}$ was calculated for K_I from the slopes. BPCA is also strictly competitive with DPN^+ at pH 10. 10mM glycine-NaOH, plus phosphate to give an ionic strength of 0.1 and a BPCA concentration of 256mM was used. K_I as calculated from the slopes is $120\mu\text{M}$.

BPCA is competitive with ethanol (fig. 7-5) and an average value of $108\mu\text{M}$ was calculated for K_I from the slopes.

Fig. 7-6 shows that mixed inhibition is obtained when DPNH was varied. The intercept is increased because of formation of EO.BPCA, while the slope increases because of competition between DPNH and BPCA. An average value for K_I of $28\mu\text{M}$ was calculated from the slopes.

BPCA is uncompetitive with acetaldehyde (fig. 7-7). This indicates that ER.BPCA is not formed and the change in intercept is due to formation of EO.BPCA.

The results show that the mode of binding of iodoacetate and BPCA is very similar, although BPCA is bound much more tightly in the ternary and binary complexes.

The interaction of BPCA with other inhibitors was investigated by the method of Theorell and Yonetani (1964).

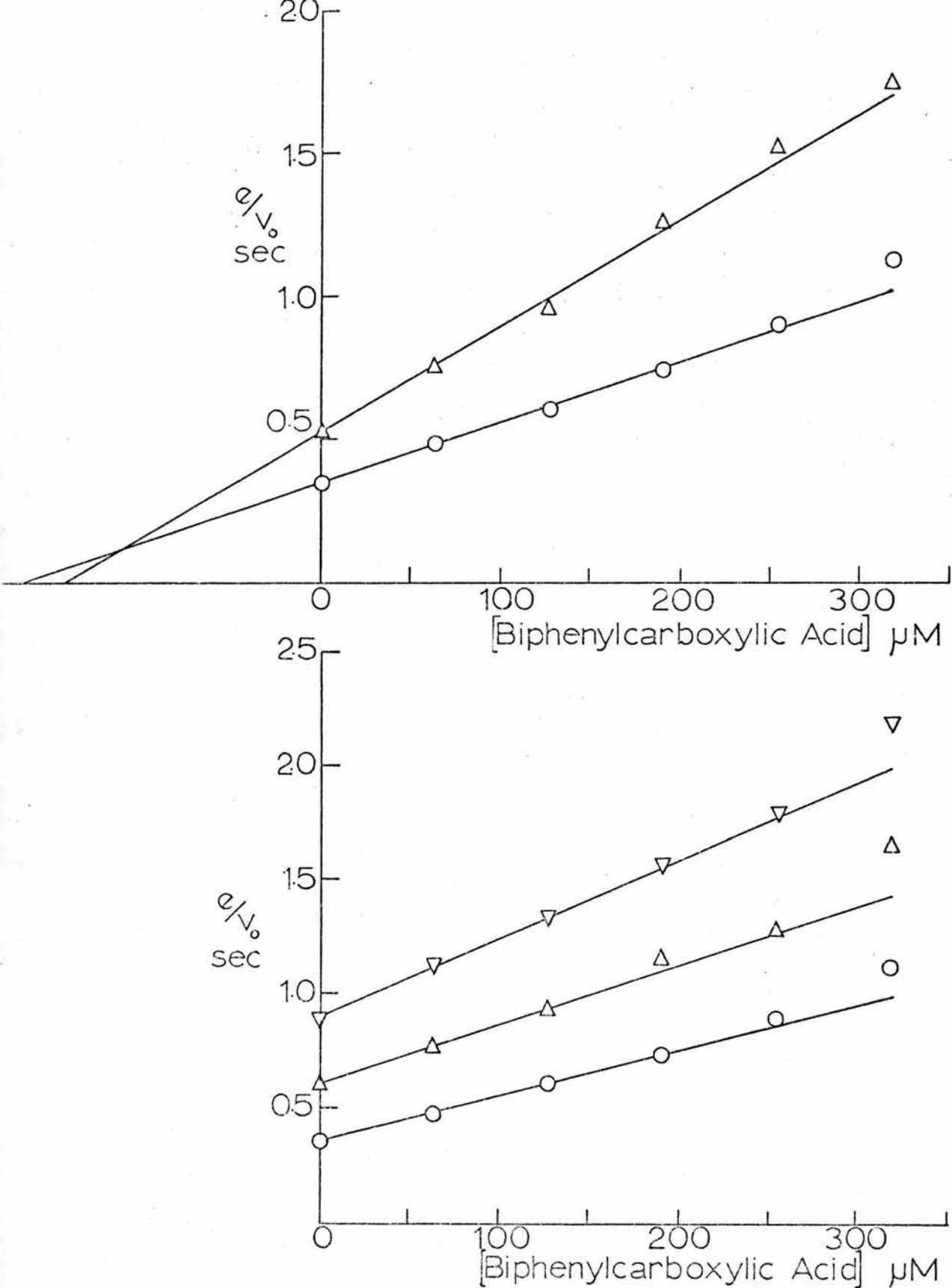


Fig.7-8. Multiple inhibition by BPCA and adenosine at constant DPN^+ ($37.6 \mu\text{M}$) and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C ; o, without adenosine; Δ , + adenosine (17.0mM).

Fig.7-9. Multiple inhibition by BPCA and AMP at constant DPN^+ ($37.6 \mu\text{M}$) and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; o, without AMP; Δ , ($260 \mu\text{M}$), ∇ , ($520 \mu\text{M}$).

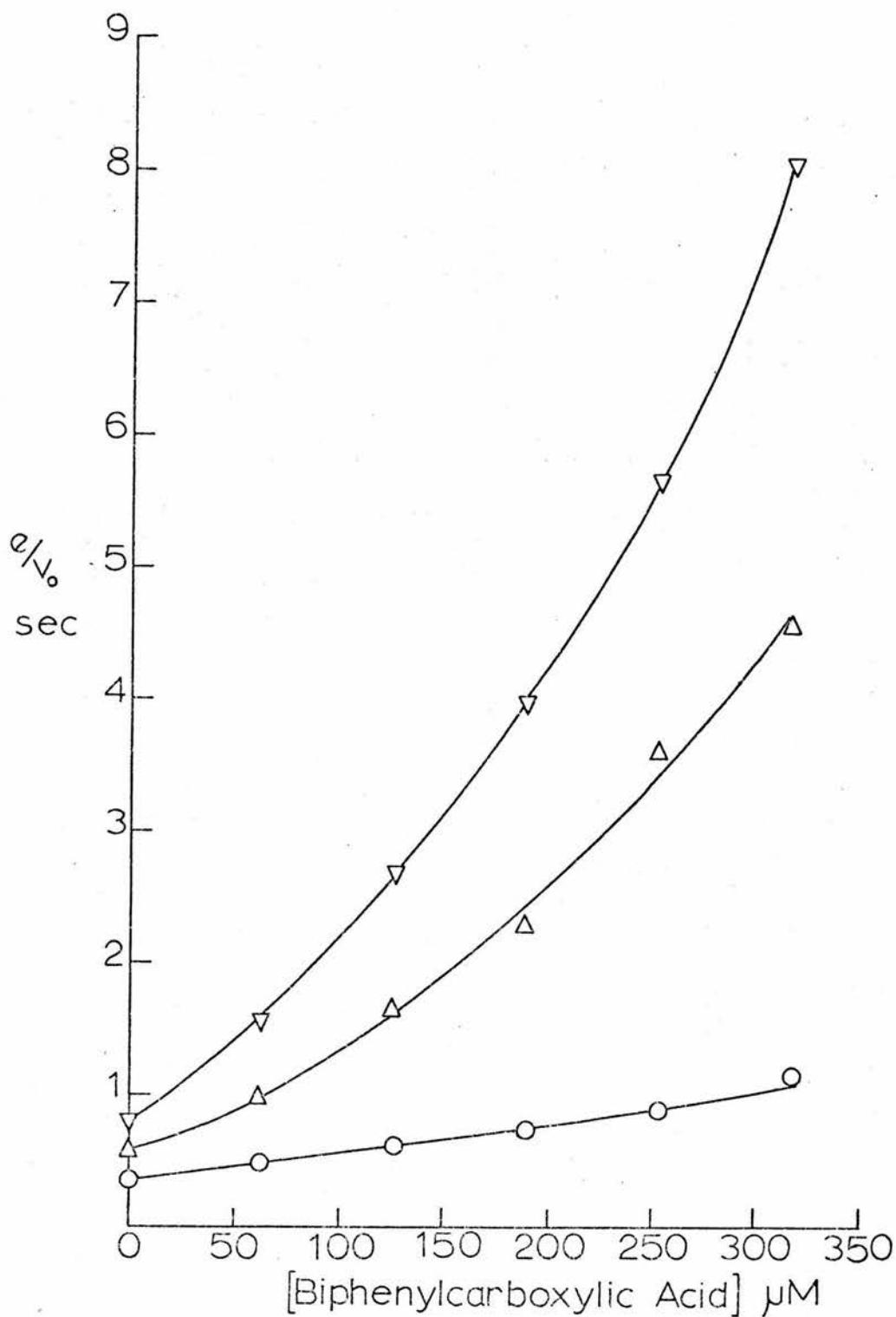


Fig.7-10. Multiple inhibition by BPCA and orthophenanthroline at constant DPN^+ ($37.6 \mu\text{M}$) and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C ; o, orthophenanthroline; + orthophenanthroline, Δ , ($15.1 \mu\text{M}$), ∇ , ($30.2 \mu\text{M}$).

Over the range of BPCA concentration used there is no obvious curvature with BPCA alone, or in the presence of adenosine (fig. 7-8), or AMP (fig. 7-9). However, in the presence of orthophenanthroline (fig. 7-10) the plots become concave-up. Increasing the range of BPCA concentration revealed that a Dixon plot (Dixon, 1953) with varying BPCA is concave-up when the DPN^+ concentration was low and ethanol concentration high. The probable explanation of this effect is that more than one BPCA molecule is bound per active centre. Previous workers (Ebersole et al., 1944; Snyder et al., 1965) have estimated the number of inhibitor molecules bound (r), by supposing the following equation to be valid;

$$v/v_i = 1 + \frac{(I)^r}{K_I} \quad (1)$$

from which it follows,

$$\log(v/v_i - 1) = r \log(I) + \log K_I \quad (2)$$

In these equations, v is the initial velocity without inhibitor, and v_i is the initial velocity with inhibitor, I . Equation (2) was applied to the data in fig. 7-11 and a straight line drawn through the points as shown in fig. 7-12. The slope of the line is 1.7 so it is probable that two BPCA molecules are bound per active centre. The inhibition of LADH by BPCA may be represented by the following scheme:

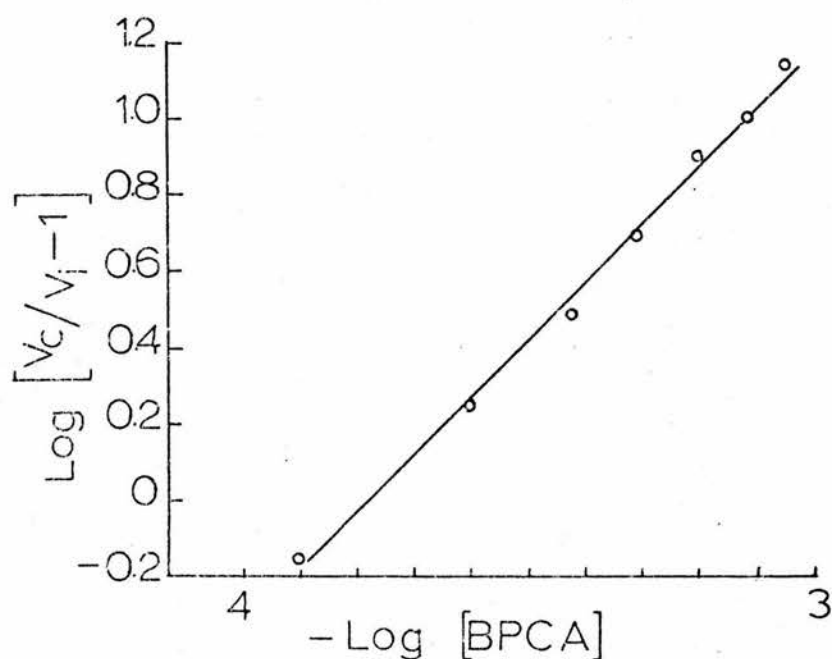
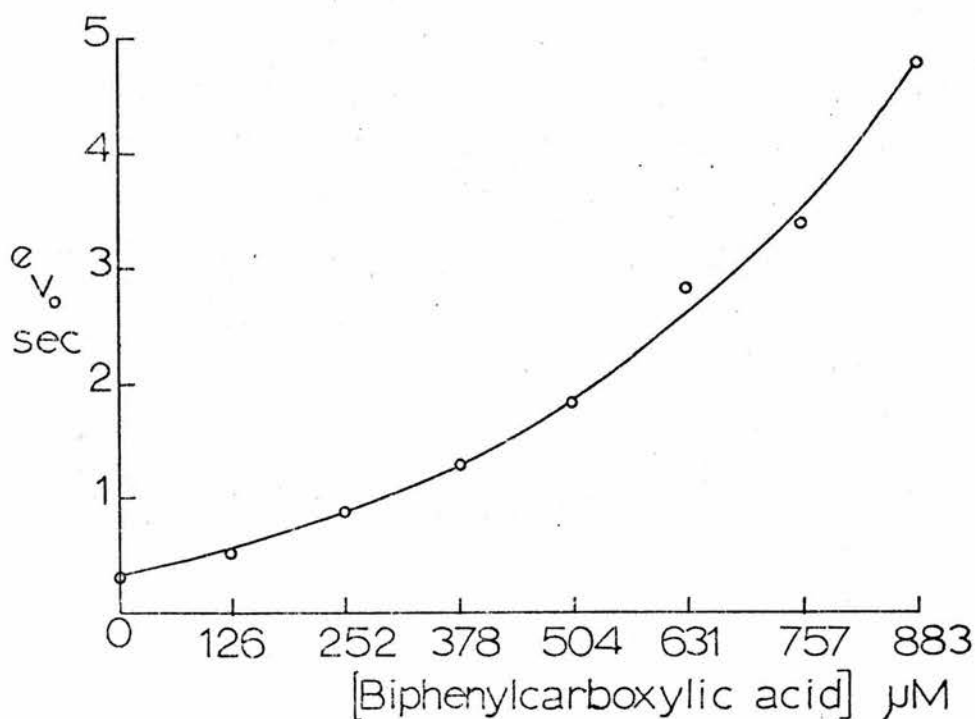


Fig.7-11. The effect of varied BPCA concentration on kinetics with constant DPN^+ ($40\mu\text{M}$) and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C .

Fig.7-12. Estimation of the number of BPCA molecules per active site involved in inhibition using the same data as in fig.7-11. (v_c is initial velocity without BPCA; v_i is initial velocity with BPCA).

$$e/v = \frac{1}{k_2} + \frac{1}{k_1(0)} \left\{ 1 + \frac{(I)}{K_1} + \frac{(I)}{K_2} + \frac{(I)^2}{\alpha K_1 K_2} \right\} + \frac{1}{k_3(\text{alc})} \left\{ \frac{1+(I)}{K_I} \right\} \\ + \frac{k_1}{k_1 k_3(0)(\text{alc})} \left\{ 1 + \frac{(I)}{K_1} + \frac{(I)}{K_2} + \frac{(I)^2}{\alpha K_1 K_2} \right\} \quad (3)$$

Thus a plot of e/v against (I) will be a quadratic function of (I) and concave-up.

Now from equation (3): as (alcohol) $\rightarrow \infty$

$$v/v_i = \frac{k_1(0) + k_2 \left\{ 1 + \frac{(I)}{K_1} + \frac{(I)}{K_2} + \frac{(I)^2}{\alpha K_1 K_2} \right\}}{k_1(0) + k_2}$$

From which it follows that;

$$\log (v/v_i - 1) = \log \left\{ \frac{(I)}{K_1} + \frac{(I)}{K_2} + \frac{(I)^2}{\alpha K_1 K_2} \right\} + \log \left\{ \frac{k_2}{k_1(0)+k_2} \right\} \quad (4)$$

Consequently equation (2) is only strictly valid if the binary complexes are not formed. The slope of $\log (v/v_i - 1)$ against $\log (I)$ will thus always be smaller than the number of binding sites which can bind I simultaneously. The slope will depend on the value of α . In scheme A, the slope of equation (2) will approach 1 as α tends to infinity and will approach 2 as α tends to zero. Thus the slope gives a comparative indication of the degree of interaction between different inhibitors.

If the interaction of BPCA with a coenzyme com-

competitive inhibitor J, is investigated, the following equilibria must be considered in addition to those already defined for scheme (A).

$$K_J = \frac{(E)(J)}{(EJ)} = \frac{(EI_1)(J)}{\beta(EI_1J)} = \frac{(EI_2)(J)}{\gamma(EI_2J)} = \frac{(EI_1I_2)(J)}{\beta\gamma(EI_1I_2J)}$$

In the presence of I and J the initial rate equation (3) becomes;

$$\frac{e}{v} = \frac{1}{k_2} + \frac{\left[1 + \frac{(J)}{K_J} + \frac{F(I)}{k_1(0)}\right]}{k_1(0)} + \frac{\left[1 + \frac{(I)}{K_I}\right]}{k_3(\text{alc})} + \frac{k_{-1}}{k_1k_3(0)(\text{alc})} \left[1 + \frac{(J)+F(I)}{K_J}\right] \quad (5)$$

Where,

$$F(I) = (I) \left\{ \frac{1}{K_1} + \frac{1}{K_2} + \frac{(J)}{K_1K_J} + \frac{(J)}{K_2K_J} \right\} + (I)^2 \left\{ \frac{1}{K_1K_2} + \frac{(J)}{K_1K_2K_J} \right\}$$

Thus a plot of e/v against (I) is still a quadratic function of (I) .

From (5),

$$e/v = a(I)^2 + b(I) + c \quad (6)$$

Where,

$$a = \left\{ \frac{1}{k_1(0)} + \frac{k_{-1}}{k_1k_3(0)(\text{alc})} \right\} \left\{ \frac{1}{K_1K_2} + \frac{(J)}{K_1K_2K_J} \right\}$$

$$b = \frac{1}{k_3(\text{alc})} \left\{ \frac{1}{K_1} + \frac{1}{K_2} + \frac{(J)}{K_1K_J} + \frac{(J)}{K_2K_J} \right\} + \frac{k_{-1}}{k_1k_3(0)(\text{alc})} \left\{ \frac{1}{K_1} + \frac{1}{K_2} + \frac{(J)}{K_1K_J} + \frac{(J)}{K_2K_J} \right\}$$

$$c = \frac{1}{k_2} + \frac{\left[1 + \frac{(J)}{K_J}\right]}{k_1(0)} + \frac{1}{k_3(\text{alc})} + \frac{k_{-1}}{k_1k_3(0)(\text{alc})} \left[1 + \frac{(J)}{K_J}\right]$$

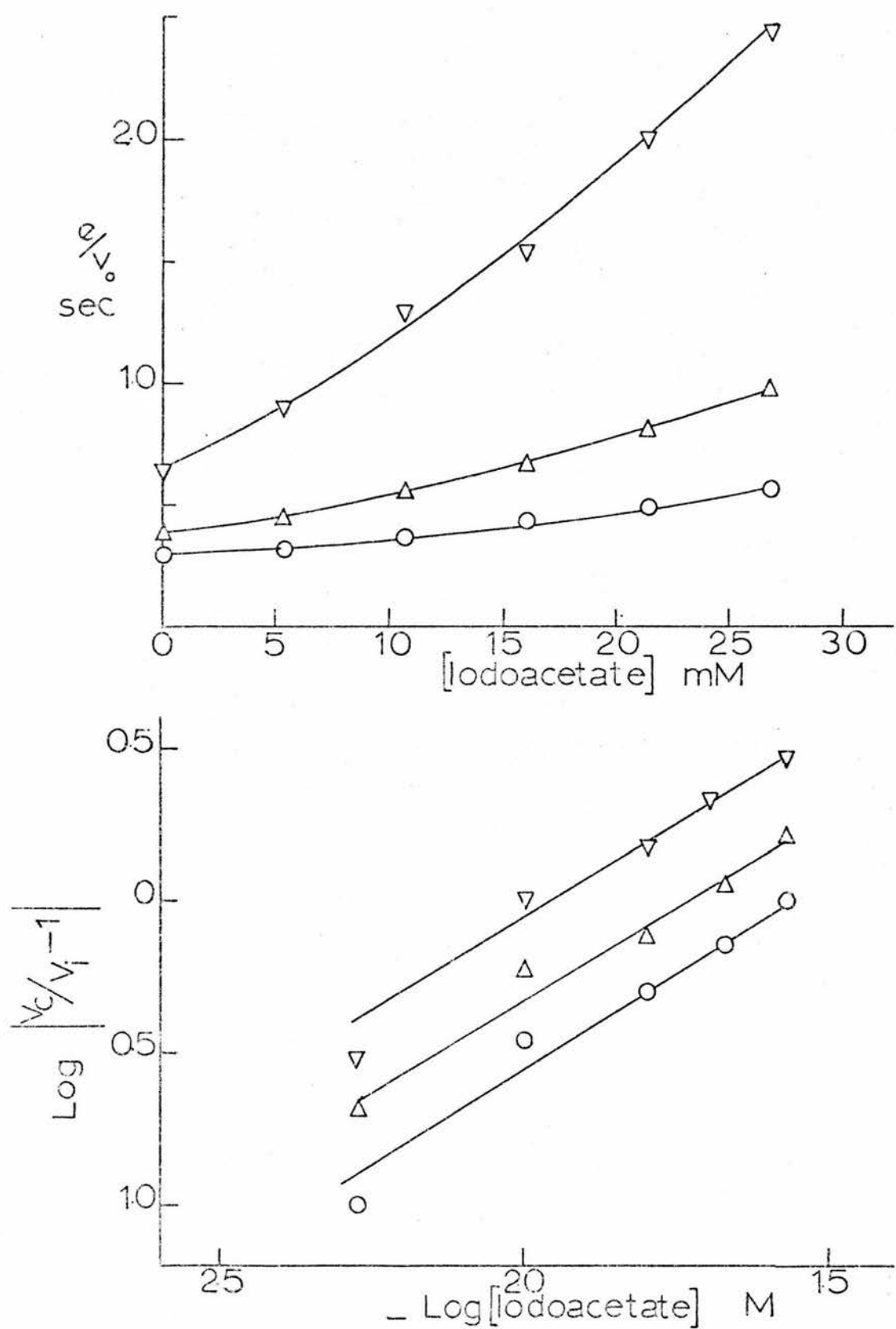


Fig.7-13. The effect of varied iodoacetate concentration on kinetics with constant DPN^+ and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C; o, DPN^+ (250 μM); Δ , DPN^+ (25 μM); ∇ , DPN^+ (6.5 μM). (v_0 is the initial velocity without iodoacetate; v_i is the initial velocity with iodoacetate).

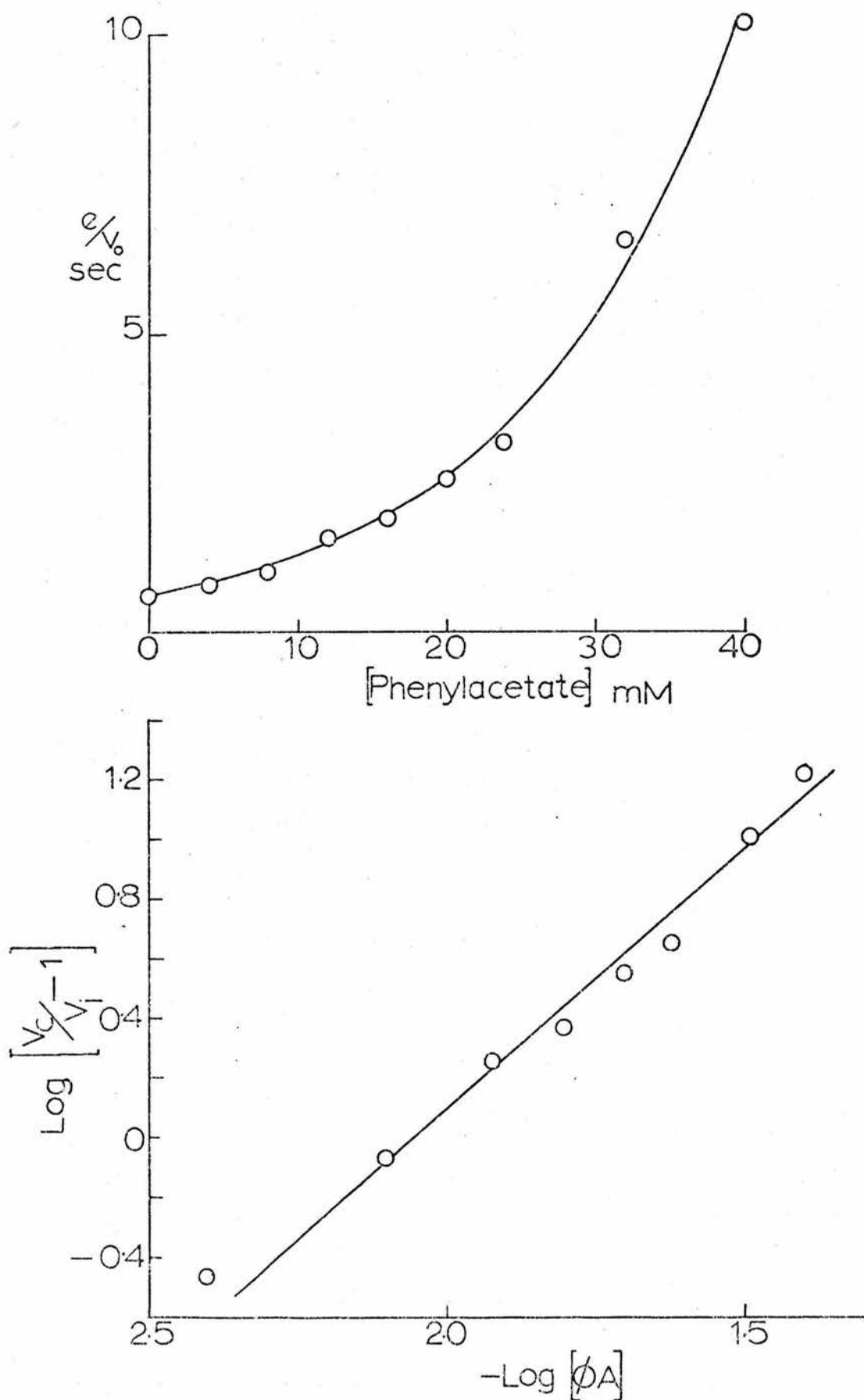


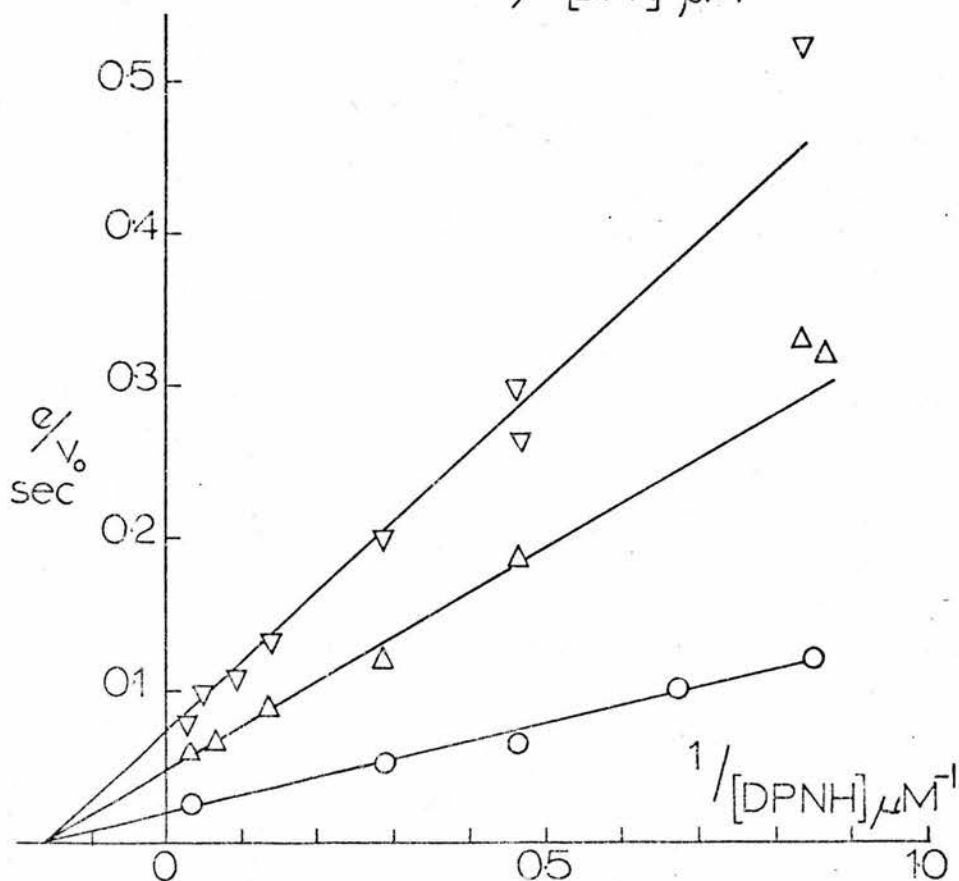
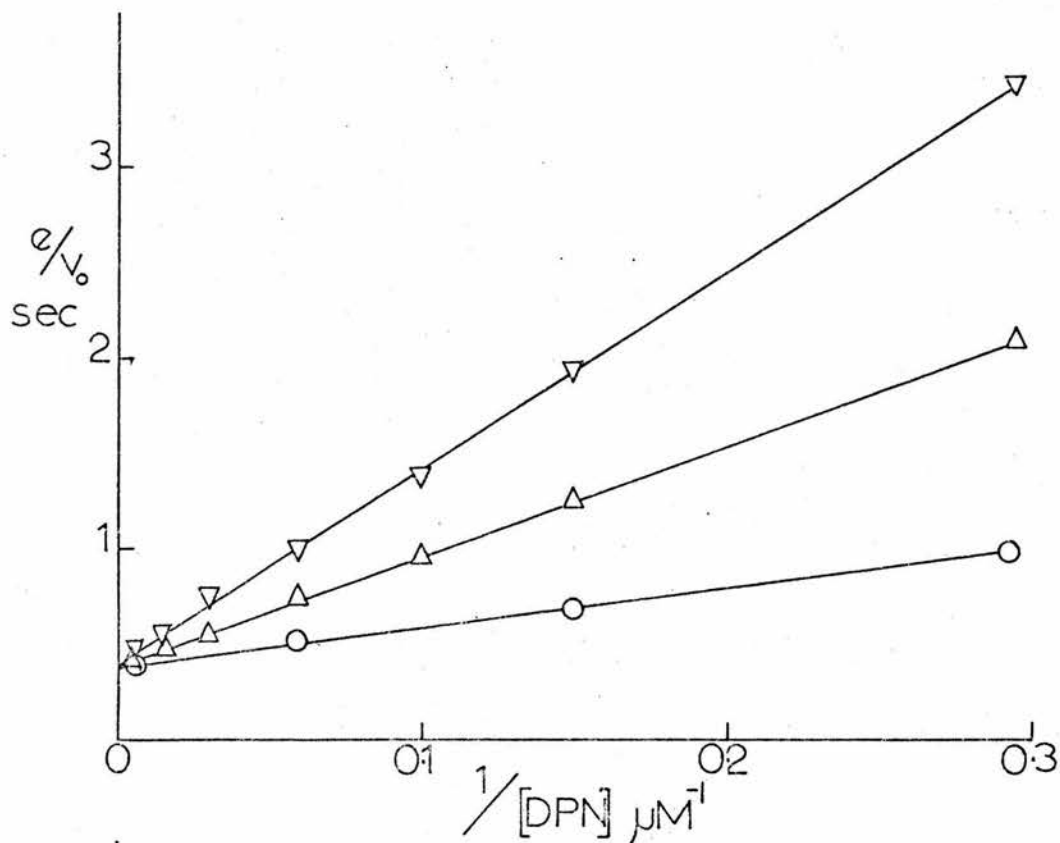
Fig.7-14. The effect of varied phenylacetic acid concentration on kinetics with constant DPN^+ ($16.8 \mu\text{M}$) and ethanol (8.25mM) concentrations. Phosphate buffer, pH 7.14, ionic strength 0.1; 23.5°C (V_0 is the initial velocity without phenylacetic acid; V_1 is the initial velocity with phenylacetic acid).

$$\text{Now, } d(e/v)/d(I) = 2a(I) + b \quad (7)$$

The above relationship (7) indicates that if β and γ have finite values, plots of e/v versus (I) in the presence of J will have a greater value of $(d(e/v)/d(I))$ at each value of (I) than in the absence of J . If both β and γ are infinite the curve in the presence of J will be the same shape as in the absence of J ($d(e/v)/d(I)$ is the same at each value of (I) as in the absence of J), but will be displaced upwards due to the presence of (J) in the term c above.

Applying this theory to the results presented in figs. 7-8, 7-9 and 7-10 the following conclusions can be drawn. AMP and BPCA interact strongly since there is very little change of slope in fig. 7-9. This indicates that AMP is competitive with BPCA at both binding sites. Fig. 7-8 indicates that at weak LADH-adenosine-BPCA complex is formed. Any interaction between orthophenanthroline and BPCA must be weak since the rate of change of slope in the presence of orthophenanthroline is greatly increased (fig. 7-10). These results agree with the findings that iodoacetate is strictly competitive with AMP, forms a weak LADH-adenosine-iodoacetate complex and is non-competitive with orthophenanthroline (Reynolds et al., 1969).

Dixon plots with varying iodoacetate (fig. 7-13) and phenylacetic acid concentrations (fig. 7-14) are concave-up. The corresponding plots of $\log (v/v_i - 1)$ against $\log (I)$ have slopes of 1.2 for iodoacetate and 1.8 for phenylacetic acid. This may indicate a greater degree of interaction between simultaneously bound iodoacetate



Kinetics in the presence of Phenylacetic acid. (Phosphate buffer, pH 7.14, ionic strength 0.1, 23.5°C).

Fig.7-15. Constant ethanol concentration (8.25mM) and varied DPN^+ concentration o, without inhibitor; + phenylacetic acid, Δ , (4.0mM), ∇ , (8.0mM).

Fig.7-16. Constant acetaldehyde concentration (2.90mM) and varied DPNH concentration o, without inhibitor; + phenylacetic acid, Δ , (4.0mM), ∇ , (8.0mM).

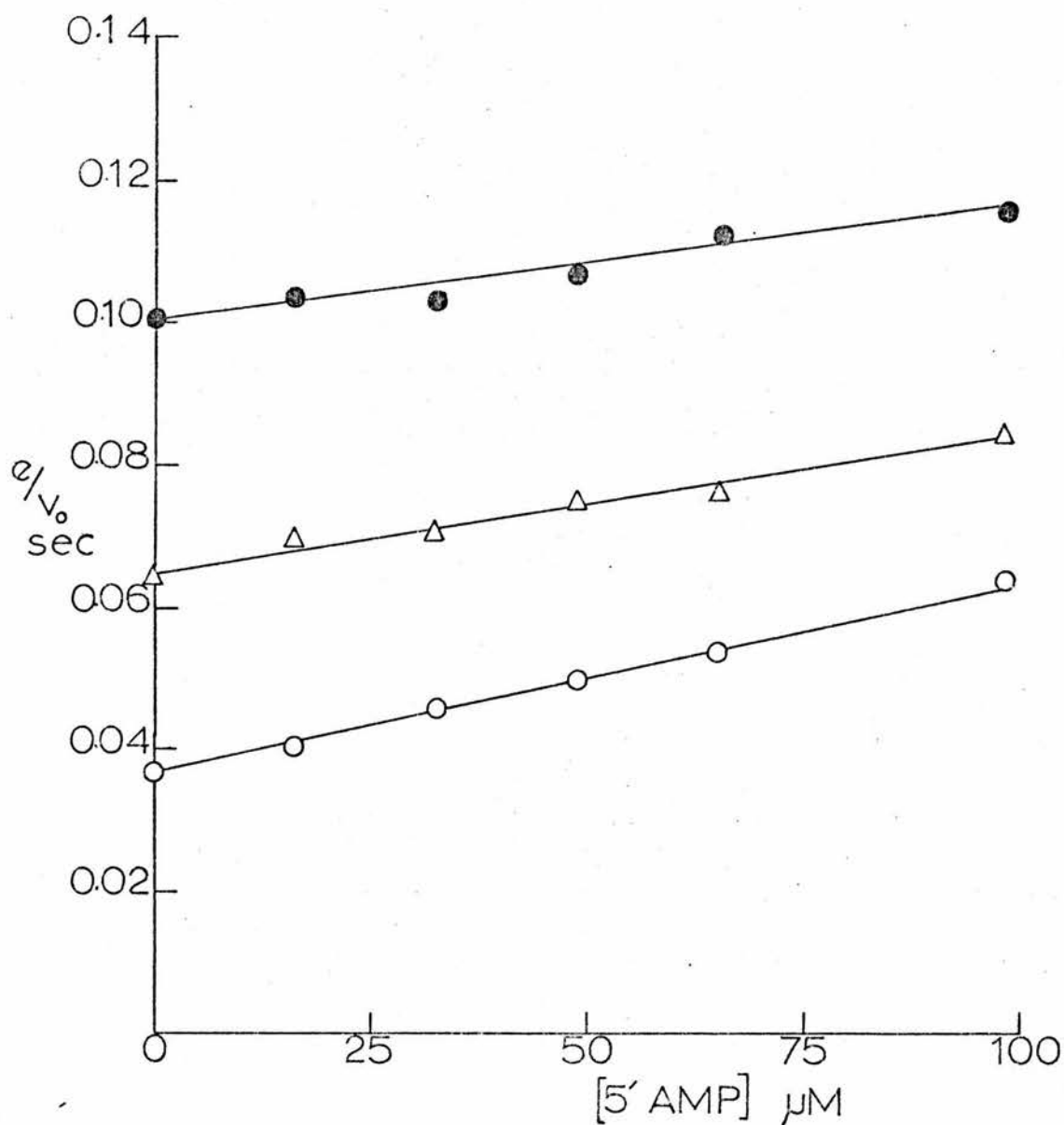


Fig.7-17. Multiple inhibition by chloride and AMP at constant DPNH ($8.1 \mu\text{M}$) and acetaldehyde (3.70mM) concentrations. Phosphate buffer, pH 7.14, (phosphate concentration was varied to maintain an ionic strength of 0.5); o, without chloride; Δ , + chloride, (0.1M), \bullet , (0.2M).

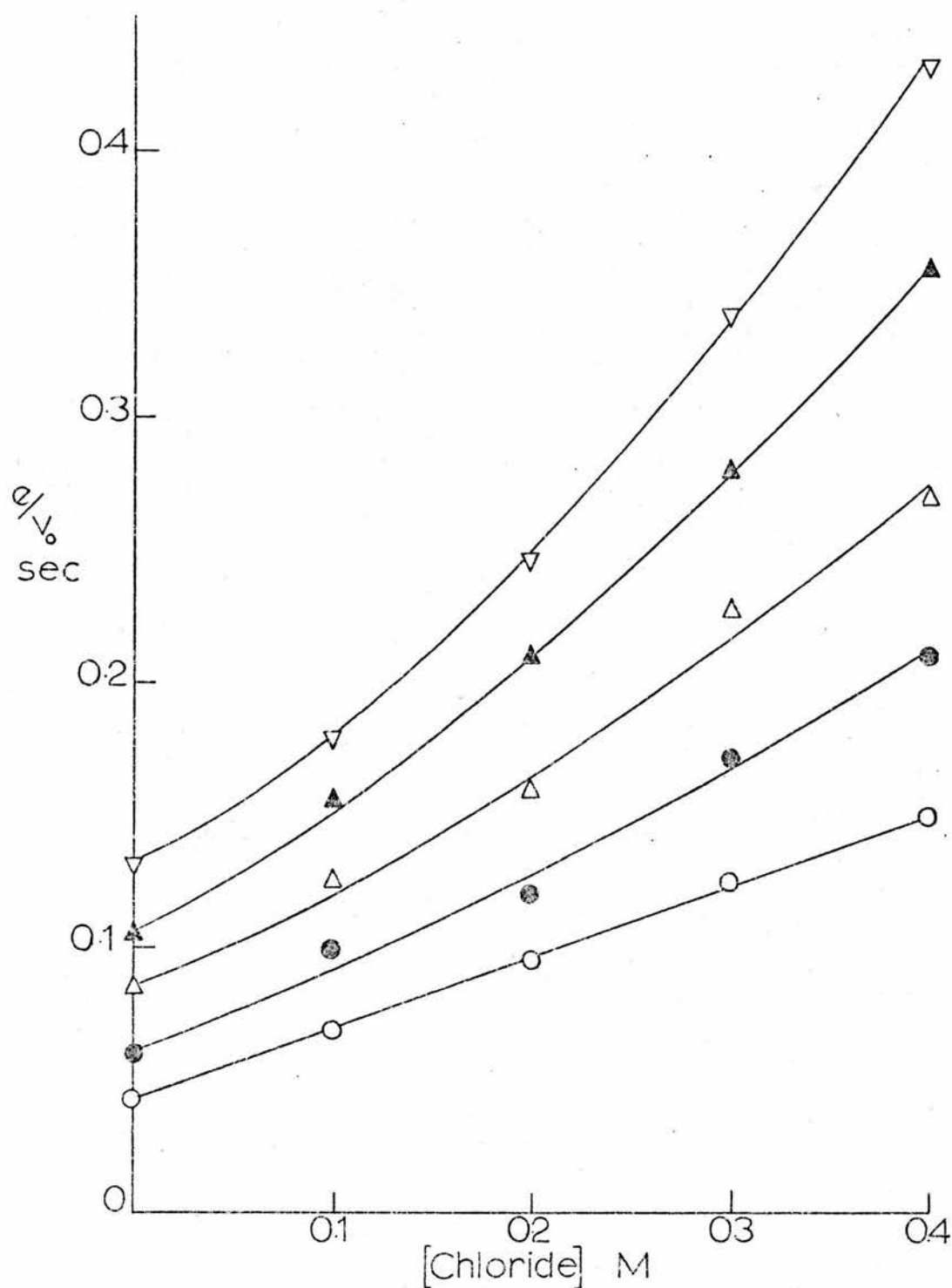


Fig.7-18. Multiple inhibition by chloride and orthophenanthroline at constant DPNH ($8.1 \mu\text{M}$) and acetaldehyde (3.70mM) concentrations. Phosphate buffer, pH 7.14, (phosphate concentration was varied to maintain an ionic strength of 0.5); ○, without orthophenanthroline; + orthophenanthroline ●, ($3.2 \mu\text{M}$), Δ, ($6.4 \mu\text{M}$), ▲, ($9.6 \mu\text{M}$), ▽, ($12.8 \mu\text{M}$).

molecules than for the aromatic acids.

Phenylacetic acid is strictly competitive with DPN^+ (fig. 7-15) and gives mixed inhibition when DPNH is varied (fig. 7-16). This indicates that phenylacetic acid is competitive with DPN^+ and DPNH and forms a ternary complex EO.phenylacetic acid. The value of K_I calculated from the slopes is 2.4mM from fig. 7-15 and 2.6mM from fig. 7-16. The additional phenyl ring of BPCA thus results in 80-fold stronger binding than phenylacetic acid.

Chloride and AMP

A Theorell-Yonetani plot (1964) showing the interaction of chloride and AMP is shown in fig. 7-17. Both inhibitors are competitive with coenzyme so the parallel plots indicate that the two inhibitors are competitive. The formation of EO.chloride does not change this interpretation.

Chloride and orthophenanthroline

A Theorell-Yonetani plot (1964) illustrating the interaction of chloride and orthophenanthroline is shown in fig. 7-18. The ionic strength of 0.5 was maintained by varying the phosphate concentration. The plots are curved-up, indicating apparent competition at low chloride concentrations becoming non-competitive at high chloride concentrations. A similar result was found when this interaction was investigated using the kinetics of iodoacetate inactivation (Reynolds et al., 1970). These results suggest chloride binds competitively with orthophenanthroline and weakly non-competitively. More than one chloride ion may be involved.

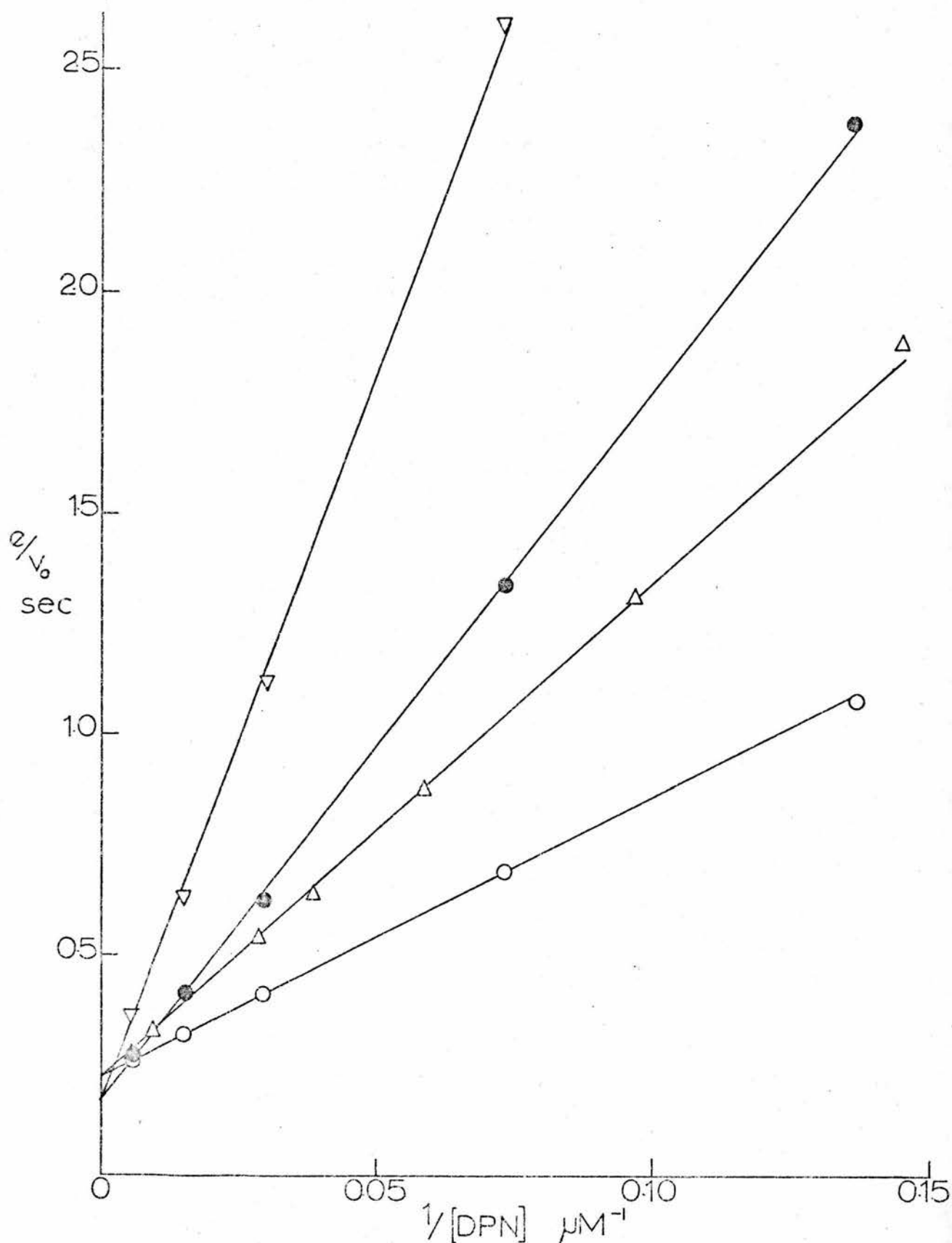


Fig.7-19. Kinetics in the presence of halides at constant ethanol concentration (8.25mM) and varied DPN^+ concentration. Buffer, 10.0mM glycine-NaOH plus phosphate (phosphate concentration was varied to maintain an ionic strength of 0.2), pH 10.0; 23.5°C; o, without halide; Δ , + chloride (125mM); \bullet , + bromide (125mM); ∇ , + iodide (125mM).

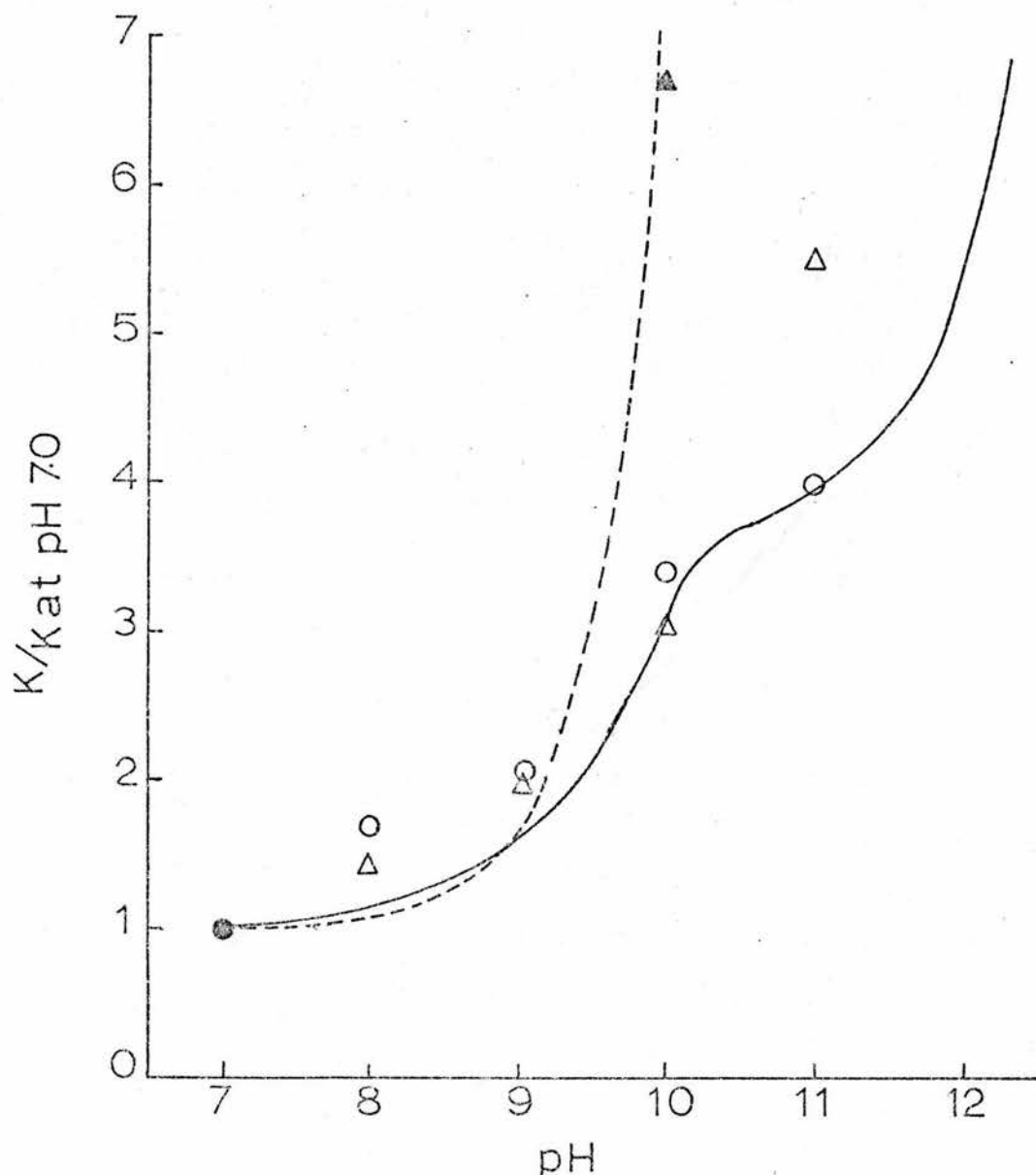


Fig. 7-20. The inhibition constants of chloride and bromide at different pH values shown as ratios of their values at pH 7.0. The inhibition constants were obtained from experiments similar to that shown for pH 10.0 in fig. 7-19, and are presented in table 7-1. The continuous line is a theoretical titration curve for binding influenced by the interaction of two pKs ($pK_1 = 9.0$ and $pK_2 = 12.5$) with a ratio of dissociation constants 1.0 at acid pH and infinity at alkaline pH. pK_1 and pK_2 represent functions contributing 75% and 25% respectively of the binding energy. Experimental points are; o, chloride; Δ , bromide. The broken line is a theoretical titration curve for a pK of 9.2 with a ratio of dissociation constants 1.0 at acid pH and infinity at alkaline pH. \blacktriangle , is an experimental point for AMP (inhibition constants from chapter 4 were used).

TABLE 7-1.

Kinetic determination at various pH values of inhibition constants (K_I) for chloride and bromide.

At pH 10.0, K_I was evaluated from the slopes in fig. 7-19. At other pH values, K_I was evaluated from analogous experiments. At pH 7.0 and pH 8.0 phosphate buffer was used; chloride (150mM) and bromide (50mM). At pH 9.0 and pH 11.0, 10mM glycine NaOH plus phosphate was used; chloride (150mM) and bromide (100mM). The ionic strength was maintained at 0.3 at all pH values.

pH	Chloride		Bromide	
	K_I (mM)	Inhibition-type	K_I (mM)	Inhibition-type
7.0	50	CS	25	CS
8.0	86	CS	35	CS
9.1	104	CS	49	CS
10.0	170	C	77	CS
11.0	206	C	138	C

CS = Competitive with stimulation at high DPN^+ concentration.

C = Strictly competitive.

The pH dependence of chloride and bromide binding

Inhibition constants were determined for chloride and bromide binding from Lineweaver-Burk plots with varying DPN^+ concentrations as shown for pH 10.0 in fig. 7-19 and as presented in table 7-1. The ionic strength was maintained at 0.3 by varying the phosphate concentration. The enhancement of initial velocity by chloride and bromide at high DPN^+ concentration due to the weakening of DPNH binding is abolished at high pH.

Fig. 7-20 shows the pH variation of the inhibition constants in table 7-1 when plotted as ratios of the inhibition constants at pH 7.0. The broken line represents a titration curve for a pK_a of 9.2 with no binding to the alkaline form and is included to illustrate that the pH dependence of the halide binding cannot be described by such a simple model. The pK_a value of 9.2 was chosen since the experimental point for AMP falls near this line. The continuous line is a theoretical curve generated as described in fig. 7-20. pK_1 was taken to be 9.0 since Reynolds and McKinley-McKee (1969) concluded that a pK_a of about 9.0 is involved in chloride binding. The highest pH value it was feasible to use in that study, however, was 9.35. pK_2 was chosen as 12.5 since this is a reasonable value for the pK_a of the guanidinium group of an arginine residue in the protein and it was suggested in chapter 5 (p.62) that PLP binding at a positively charged arginine residue may have a strong influence on PLP binding at the active centre. The binding of halide to the alkaline form of ionizing groups is not considered to

be the cause of the unexpectedly small change in the strength of halide binding at high pH values. In comparison AMP binding is weakened relatively much more than halide binding by increasing the pH from 7 to 10. Moreover, adenosine is bound much more weakly than AMP and the weak adenosine binding may be an indication of the strength of AMP binding at high pH where there is no electrostatic attraction between the phosphate group of AMP and the active centre lysine.

Although it cannot be claimed that the theoretical curve in fig. 7-20 is the explanation of the pH dependence of halide binding it is representative of a range of simple models involving more than one ionizable group on the protein and is a more reasonable fit to the experimental points than any curve describing a single ionization.

Discussion

The kinetic results indicate that iodoacetate, BPCA and phenylacetic acid inhibit LADH in a similar manner. They all form EOI complexes and are strictly competitive with coenzyme. It seems therefore, that simple aromatic acids form ternary complexes, similar to those involving decanoate in which the carboxylate group is thought to lie between the positively charged zinc atom and the positively charged nicotinamide ring nitrogen of the coenzyme (Theorell and McKinley-McKee, 1961). The aromatic ring system will bound at the same hydrophobic slot as the aliphatic chain of decanoate. It was also found that there are apparently two binding sites per active centre for these inhibitors, and it is suggested that binding at both these sites is competitive with inhibitor. Phenylacetic acid is bound 80-fold more weakly than BPCA at pH 7.14, probably because the binding site is a hydrophobic region. It was found that BPCA is competitive with AMP but a weak LADH-adenosine-BPCA complex can be formed. It is thus likely that the hydrophobic part of these inhibitors, the aromatic rings or iodine atom, binds at the adenosine site, which is thus the hydrophobic region mentioned above. The active centre lysine would thus be located adjacent to this "pocket". The binding of the phosphate of AMP at this positive centre would prevent BPCA binding. However, when adenosine is bound the carboxylate group of BPCA will still be able to bind near the lysine although binding will be considerably weakened due to competition between adenosine

and the aromatic rings for the hydrophobic "pocket".

BPCA and orthophenanthroline appear to bind non-competitively which agrees with the finding by Sigman (1967) that BPCA did not compete with 2,2-bipyridyl for the active centre zinc. This study, however, shows that BPCA and iodoacetate are probably bound to zinc in the presence of coenzyme. Sigman (1967) found that the ternary EO.BPCA complex was not formed.

In chapter 5 it was postulated that there are two binding sites for PLP in the vicinity of the active centre, such that binding of PLP could not occur simultaneously at both sites. The finding that two molecules of BPCA iodoacetate or phenylacetic acid are competitive with DPN^+ is support for this theory. The reason that two molecules of these inhibitors but not of PLP, bind simultaneously can be explained by the greater distance between bound carboxylate groups of the acids, than between the charged phosphate groups of a PLP molecule bound as a Schiff base at the active centre lysine residue and a PLP molecule at the second site. The second site may be near the hydrophobic adenosine binding site so that it is shared by the two simultaneously bound iodoacetate or aromatic acid molecules. In the case of the aromatic acids overlapping of the ring systems may occur without such weakening of binding. The iodine atoms of iodoacetate may not be able to bind simultaneously without a greater degree of interference. This may explain why there is apparently more interaction between iodoacetate molecules than between the aromatic acids.

Several other aromatic acids have been reported to inhibit LADH. These are phenanthroic acid (Sigman, 1967), thyroxine derivatives (McCarthy et al., 1968; Gilleland and Shore, 1969), thyroacetic acid (McCarthy et al., 1968; McCarthy and Lovenberg, 1969) and salicylic acid (Dawkins et al., 1967). These inhibitors are characterized by being competitive with adenine nucleotides and non-competitive with orthophenanthroline or 2,2'-bipyridyl, although triiodothyroacetic acid weakens orthophenanthroline binding (McCarthy and Lovenberg, 1969). There was no evidence produced by the above studies to suggest that more than one of these inhibitors was bound per active centre. However, it has recently been shown that porcine lactate dehydrogenase (isoenzyme 5) is inhibited by the binding of two salicylate molecules per active centre, which are competitive with DPN⁺ (Cheshire and Park, 1972).

The pH dependence of halide binding shows that chloride and bromide behave similarly. At least two ionizing groups appear to be involved, one with a pK of about 9.0 and the other with a pK greater than 12.0. The only group with a pK greater than 12.0 in LADH is the guanidinium function of arginine. It was suggested in chapter 5 that an arginine residue may be the binding site at which PLP protects the active centre lysine. The pH dependence of halide binding gives some support to this proposition. The pK of about 9.0 is similar to that involved in the binding of AMP, (chapter 4) and iodoacetate, chloride and formate (Reynolds and McKinley-McKee, 1969).

This pK_a may represent interaction between zinc and the active centre lysine.

The binding of BPCA was weakened 3.7-fold when the pH was increased from 7.14 to 10.0 which is considerably less than the corresponding 6.7-fold weakening which occurs on AMP binding. It may be that the binding of the hydrophobic biphenyl moiety provides a greater proportion of the BPCA binding energy than is supplied by the adenosine moiety of AMP. In both cases this contribution is pH-independent. It is possible that the second binding site for BPCA could be the arginine residue implicated in the binding of halides and in PLP inactivations. In this case BPCA binding may have a pH dependence similar to the halides.

Halide binding becomes stronger as the size and polarizability of the anions increase. The K_I values are in the decreasing order chloride, bromide, iodide. This may be due to the larger anions fitting more snugly into the adenosine "pocket". Binding is also probably taking place at the active centre zinc atom and lysine residue. This is suggested by the mixture of competitive and non-competitive interaction of chloride with orthophenanthroline, and the binding of Reynolds et al., (1970) that AMP is more competitive with chloride than decanoate which binds at the active centre zinc atom but not at the active centre lysine. The Dixon plots with varying chloride indicate that only one chloride ion can bind per active centre, thus the K_I measured by competition with DPN^+ , although behaving as a single dissociation constant, is

contributed to by ions binding at more than one site.

The finding in chapter 5 that chloride and bromide increase the incorporation of PLP into LADH suggests that these anions can produce conformational changes in the enzyme. Halide ions producing conformational changes binding at sites other than the active centre may cause the weakening of DPNH binding observed in the presence of halides. Evidence for other sites comes from the work of Zeppezauer et al., (1969) who discovered that the broadening of the NMR peak of ^{81}Br in the presence of LADH was not quenched by DPN^+ , DPNH or oxyquinoline. Also, although the broadening of the NMR peak of ^{35}Cl is diminished by coenzyme it is not abolished (Lindman et al., 1970).

CHAPTER 8.

The Reaction of Diethylpyrocarbonate with Histidine in Liver Alcohol Dehydrogenase

Introduction

LADH contains seven histidine residues per subunit (Theorell et al., 1966; Jornvall and Harris, 1970) which may fulfil one or more of several possible functions.

- 1) Histidine may help to bind substrate and/or co-enzyme (Ringold, 1966).
- 2) Histidine may serve as a proton sink or source in a simple concerted mechanism for the enzyme, where it is responsible for both coenzyme and substrate activation (Ringold, 1966).
- 3) Histidine may activate cysteine-46 in a similar manner to that envisaged for the activation of thiol in yeast alcohol dehydrogenase (Rabin and Whitehead, 1962).
- 4) Histidine may serve as a ligand to zinc. Evidence has been presented that three imidazole ligands provide the best model for the environment of the active centre zinc in the enzyme (Theorell and McKinley-McKee, 1961 c; Plane and Long, 1963).
- 5) Histidine may be important for maintaining the tertiary structure of the enzyme.

Previous studies of histidine in LADH

Histidine in LADH has not previously been uniquely modified since the method employed, photooxidation, also oxidized tryptophan and tyrosine to a significant extent.

Robinson and Stoller (1962) found that after two histidines per enzyme molecule had been destroyed the enzyme was still fully active with DPN⁺ as coenzyme, but only 80% activity remained with an acetylpyridine

derivative of DPN⁺. It was also found that the rate of activity loss due to photooxidation varied with the co-enzyme analogue used to follow loss of activity. This was thought to be due to conformational change and not to direct participation by histidine in catalysis (Robinson et al., 1963).

Histidine in other dehydrogenases

Glyceraldehyde-3-phosphate dehydrogenase

In the rabbit muscle enzyme histidine-38 is essential for the maximum rate of deacylation of S-acetyl enzyme, dehydrogenase, arsenolysis and phosphatase activities (Bond and Park, 1967; Halcomb et al., 1968).

Ovadi et al., (1969) using DEP found that pig muscle enzyme is completely inactivated when 4-carbethoxyl-histidyl bonds per subunit are formed in the absence of substrates. Their results suggest that the carbethoxylation of these histidine residues altered the protein conformation.

α-Glycerolphosphate dehydrogenase

Photooxidation of the enzyme produced inactivation dependent on groups with a pK of 7.3 thought to be imidazole groups. Chemical modification with diazo-1-H-tetrazole led to complete inactivation when 2.14 histidines per enzyme molecule had been converted to the mono-azoderivative (Apitz-Castro and Suavez, 1970).

6-Phosphogluconate dehydrogenase

Rippa and Pontremoli (1968) found that photo-oxidation of the enzyme from *Candida Utilis* led to 90% activity loss when less than two histidines, out of the

thirteen present in the enzyme, had been destroyed.

Malate dehydrogenase

There are two essential histidine residues per enzyme molecule which are reactive towards iodoacetamide (Anderton, 1970; Anderton and Rabin, 1970).

Lactate dehydrogenase

This enzyme contains one histidine per subunit which is essential for enzymic activity (Woerkaus et al., 1969). It had been previously suggested by Winer and Schwert (1959) that a histidine residue in the enzyme supplies the proton which is necessary for the conversion of pyruvate to lactate.

Thus there are several dehydrogenases which contain histidines which are considered essential for activity or play an important role towards maintaining the fully functional enzyme. The use of a reagent capable of selectively modifying histidine residues in LADH might reveal whether histidine plays an essential role in the enzyme.

It has been shown (Ovadi et al., 1967) that DEP is capable of reacting specifically with histidyl residues in a variety of proteins at pH 6.0. DEP has since been used to study histidine in glyceraldehyde-3-phosphate dehydrogenase (Ovadi and Keleti, 1970), arginine kinase (Pradel and Kassab, 1968), phosphofructokinase (Setlow and Mansour, 1970), arginine oxidase (Thomé-Beau et al., 1971) and octopine dehydrogenase (Huc et al., 1971).

Experimental

Carbethoxylation of LADH and model compounds was

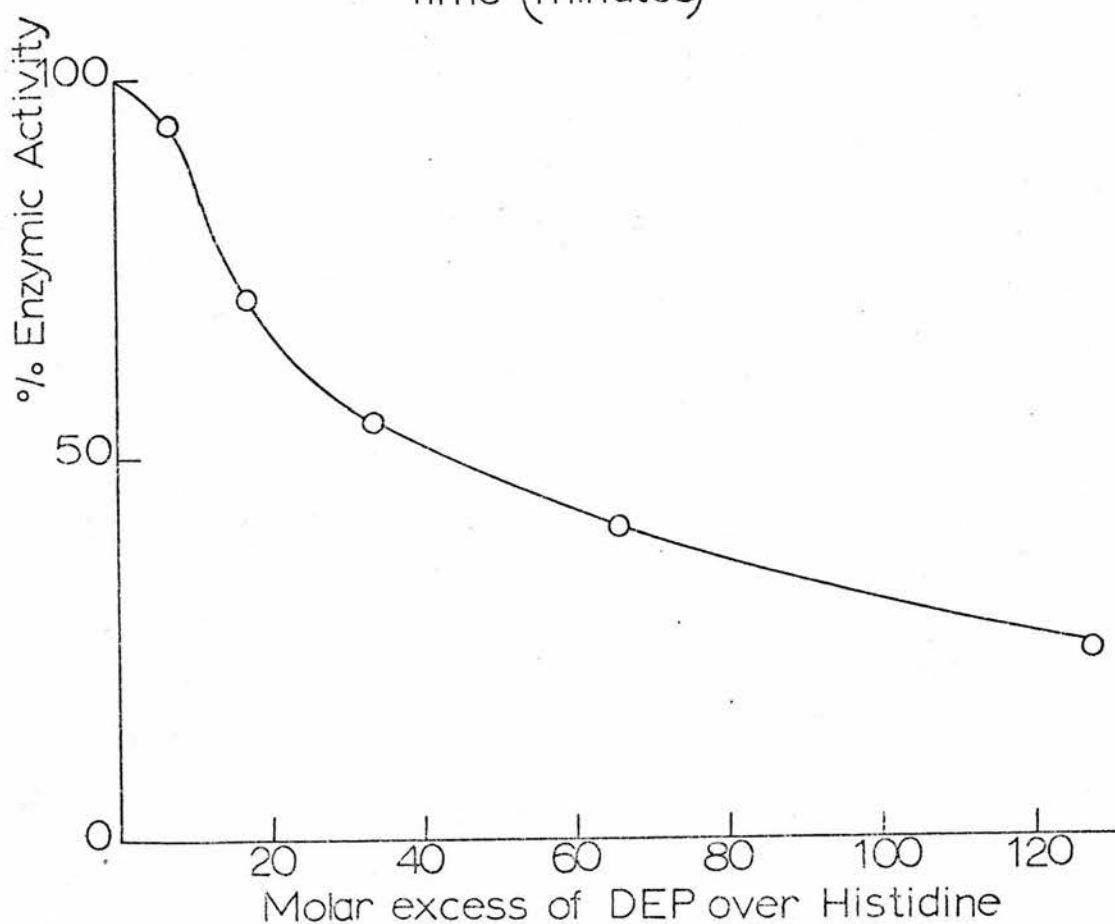
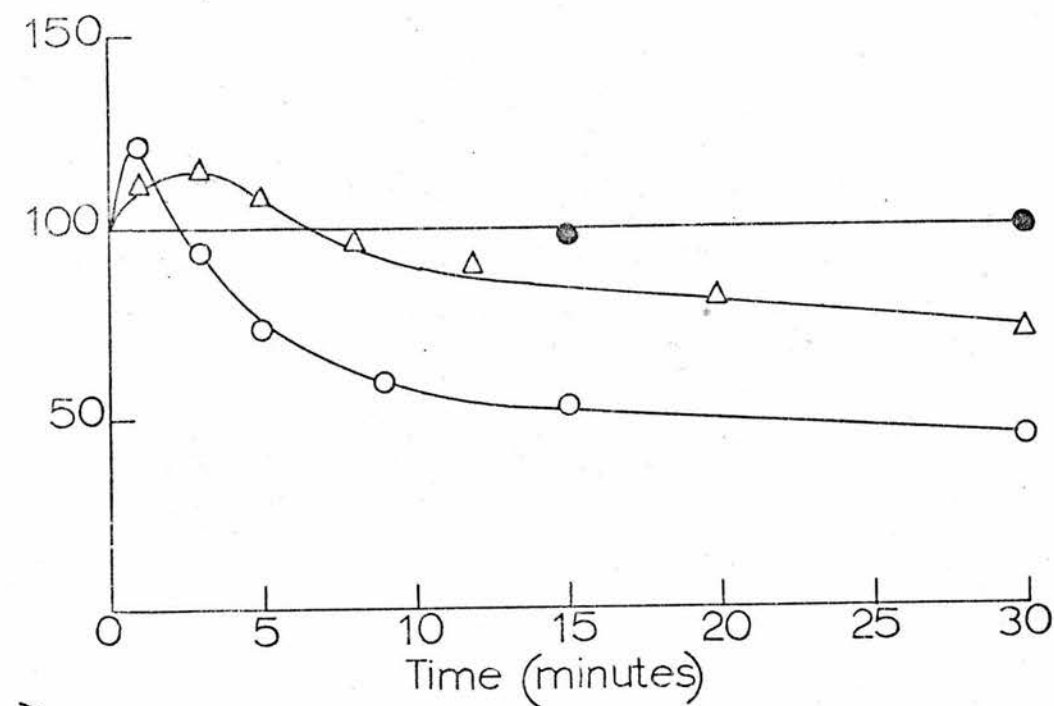


Fig.3-1. The inactivation of LADH by DEP. Enzyme, 1.80 μ M; phosphate buffer, pH 6.1, ionic strength 0.1; 23.5°C; Δ , + 520 μ M DEP (21-fold excess over the histidine content of LADH); \circ , + 130 μ M DEP (85-fold excess); \bullet , control without DEP.

Fig.3-2. The effect of the molar excess of DEP (with respect to the histidine content of LADH) on the extent of inactivation of LADH. Enzyme, 1.80 μ M; phosphate buffer, pH 6.1, ionic strength 0.1; 23.5°C. Activity was measured after incubation for 30 minutes.

carried out in 0.1M phosphate buffer, pH 6.0. DEP was added as a solution in absolute alcohol so that the final concentration of ethanol in the inactivation solution was below 2%. The number of carbethoxyl groups incorporated into histidine residues was determined by difference spectrophotometry using a millimolar extinction coefficient of 3.2 at 240nm (Ovadi et al., 1969). Throughout this chapter molar excess of DEP refers to the histidine content of the enzyme.

Enzymic activity during the course of inactivation was determined by transferring aliquots to Dalziel assay solutions and measuring the initial rate of DPNH production on the recording fluorimeter described in chapter 2. The activity is expressed as a percentage of the initial rate produced by a control solution.

Results

Effect of DEP on the enzymic activity of LADH

On incubation of LADH with DEP there is an initial rapid increase in enzymic activity followed by a slower decrease as shown in fig. 8-1. The decrease in activity is complete after incubating for about an hour, the residual activity being dependent on the molar excess of DEP used. The relationship between extent of inactivation and molar excess of DEP is shown in fig. 8-2. The extent of inactivation is temperature dependent since a 50-fold excess of DEP produced 50% inactivation at 21°C but only 15% at 0°C.

The difference spectrum of carbethoxylated LADH is shown in fig. 8-3. Spectrum -1 was generated in the

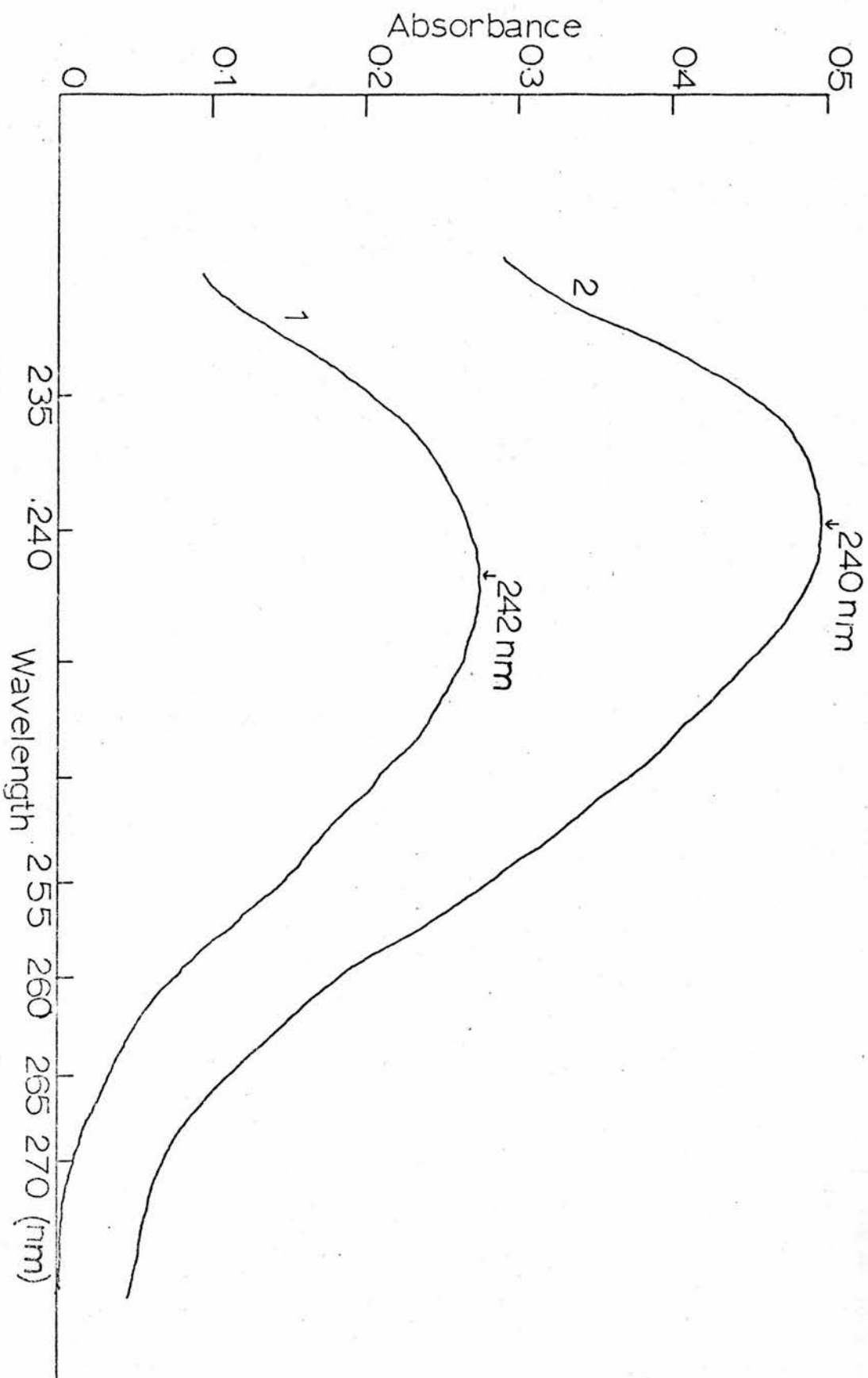


Fig. 3-3 Difference spectra of LADH modified by DEP. Enzyme ($6.6\mu\text{M}$) was incubated with DEP (9.2mM) for 20 minutes in 0.1M phosphate buffer, pH 6.1, at 20°C and spectrum-1 was measured. A further addition of DEP (9.2mM) followed by 20 minutes incubation resulted in spectrum-2.

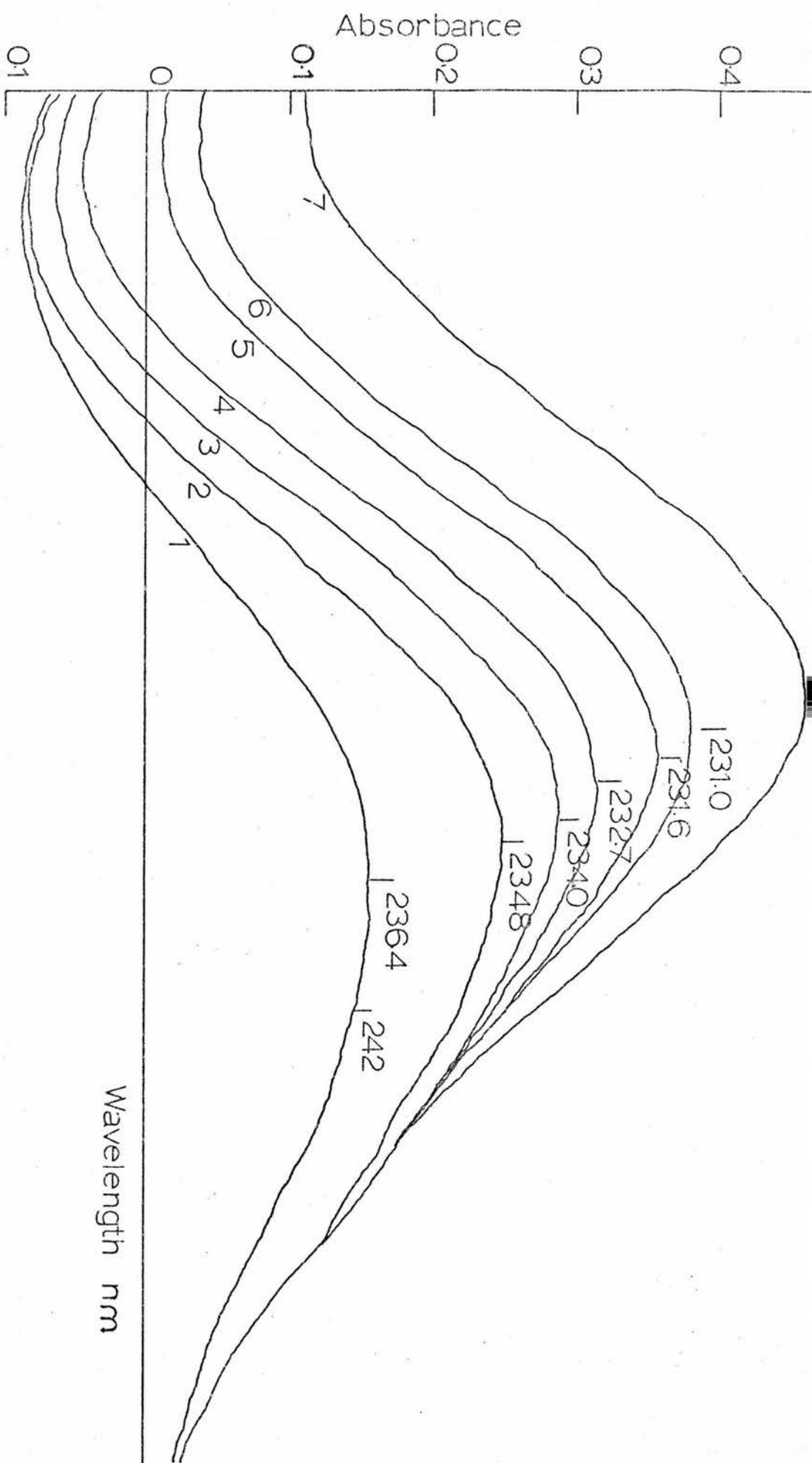


Fig. 8-4. The effect of DEP concentration on the difference spectrum of the product of the reaction between DEP and glycyl-histidine. Phosphate buffer, pH 6.1, ionic strength 0.1; 20°C; glycyl-histidine, 75.1 μ M. Successive additions of DEP were made and the solution incubated for 20 minutes after each addition. Spectra 1-4: 1.15mM DEP per addition. Spectra 3-6: 2.30mM DEP per addition. Spectrum 7 resulted from incubating for 16 hours after the final addition.

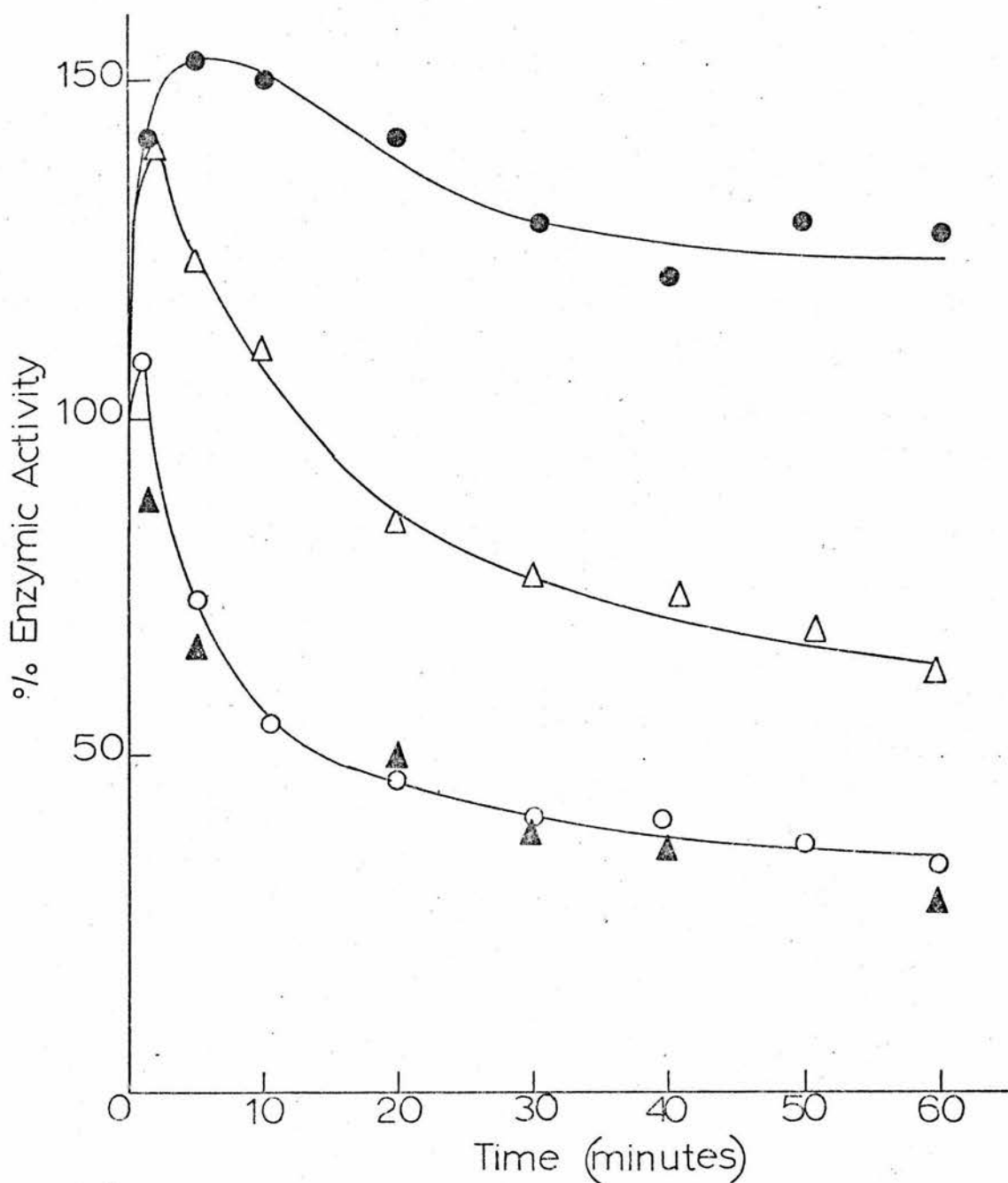


Fig.8-5. The effect of ligands on the inactivation of LADH by DEP. Enzyme, $1.80\mu\text{M}$; phosphate buffer, pH 6.1, ionic strength 0.1; 23.5°C ; DEP, 1.72mM ; o, without added ligand; Δ , + AMP (2.62mM); \bullet , + DPNH ($52\mu\text{M}$); \blacktriangle , + bromide (0.8M , giving an ionic strength of 0.9).

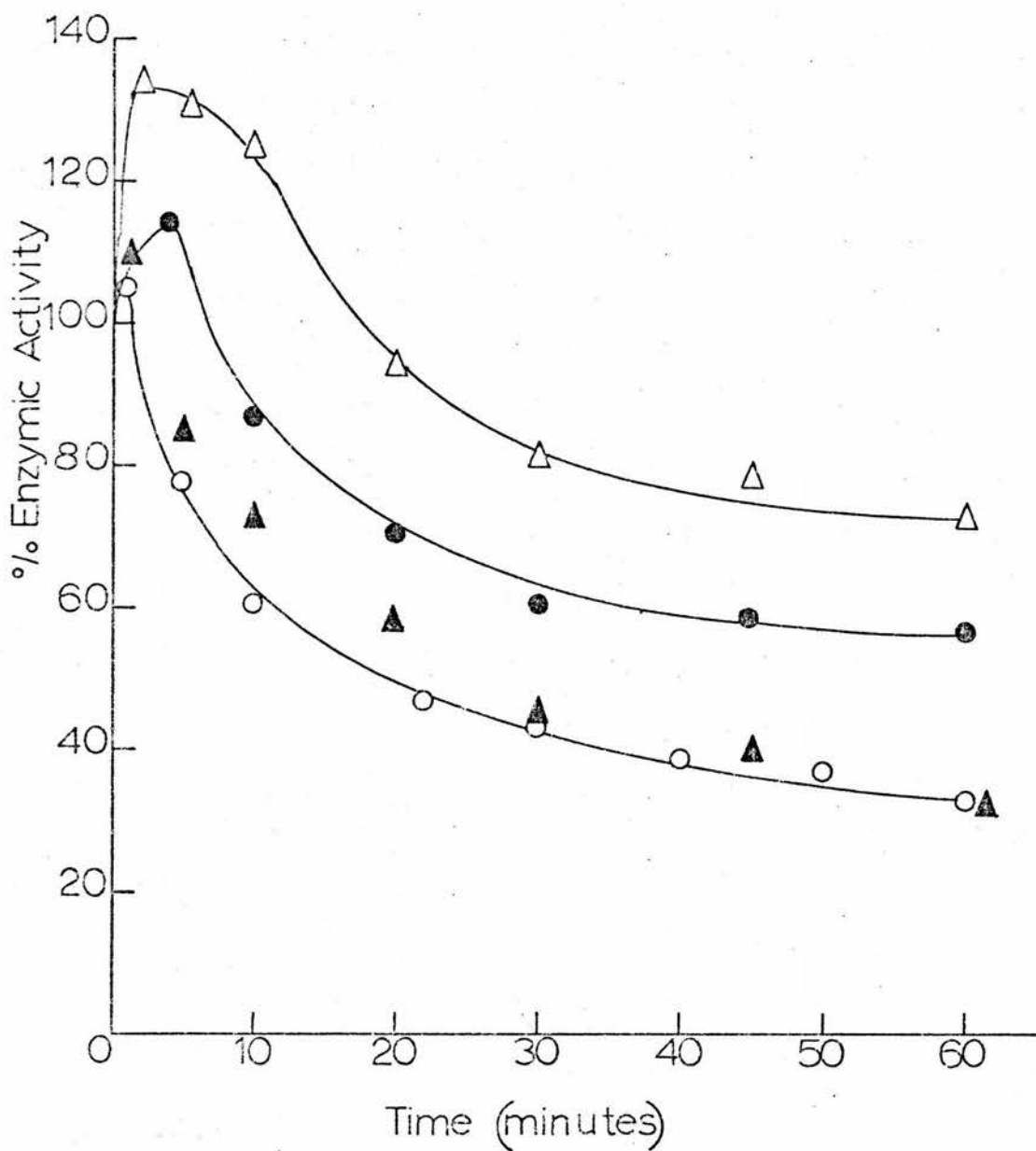


Fig.8-6. The effect of ligands on the inactivation of LADH by DEP. Enzyme, $1.80\mu\text{M}$; phosphate buffer, pH 6.1, ionic strength 0.1; 23.5°C ; DEP, 1.72mM ; o, without added ligand; ▲, + orthophenanthroline (2.43mM); ●, + decanoate (2.80mM); △, + BPCA (2.43mM).

presence of a 100-fold molar excess of DEP and has a maximum absorbance at 242nm characteristic of N-carbethoxyl histidine in proteins (Ovadi et al., 1967). However, incubation with further excess DEP led to a shift in the maximum to 240nm. There was also an increase in absorbance at 242nm to a value greater than that predicted for carbethoxylation of the entire histidine content of the enzyme.

Fig. 8-4 shows the effect of increasing DEP concentrations on the difference spectrum of glycylhistidine. The absorbance of the carbethoxylated glycylhistidine at the maximum of spectrum -1 (30-fold molar excess of DEP) is equivalent to millimolar extinction coefficient of 3.2 as found by Ovadi et al., (1967) when a 5 to 10-fold molar excess of DEP was used. Moreover, the absorption maximum shifted from 236.4nm to 229.6nm as the DEP concentration was increased. Vliegenthart and Dorland (1970) have shown that at pH 8.0 the imidazole ring of histidine is cleaved by excess DEP. A similar reaction may be causing the effects observed in fig. 8-3 and fig. 8-4. α -N-acetyl histidine was found to give the same result. Thus, to determine the extent of carbethoxylation of histidine in LADH the molar excess of DEP was kept well below 30-fold.

Fig. 8-5 shows that DPNH did not prevent activation but protected completely against inactivation. AMP protected partially against inactivation whereas bromide and high ionic strength had no effect. Fig. 8-6 shows that decanoate and 4-biphenylcarboxylic acid protected partially, while orthophenanthroline had no effect.

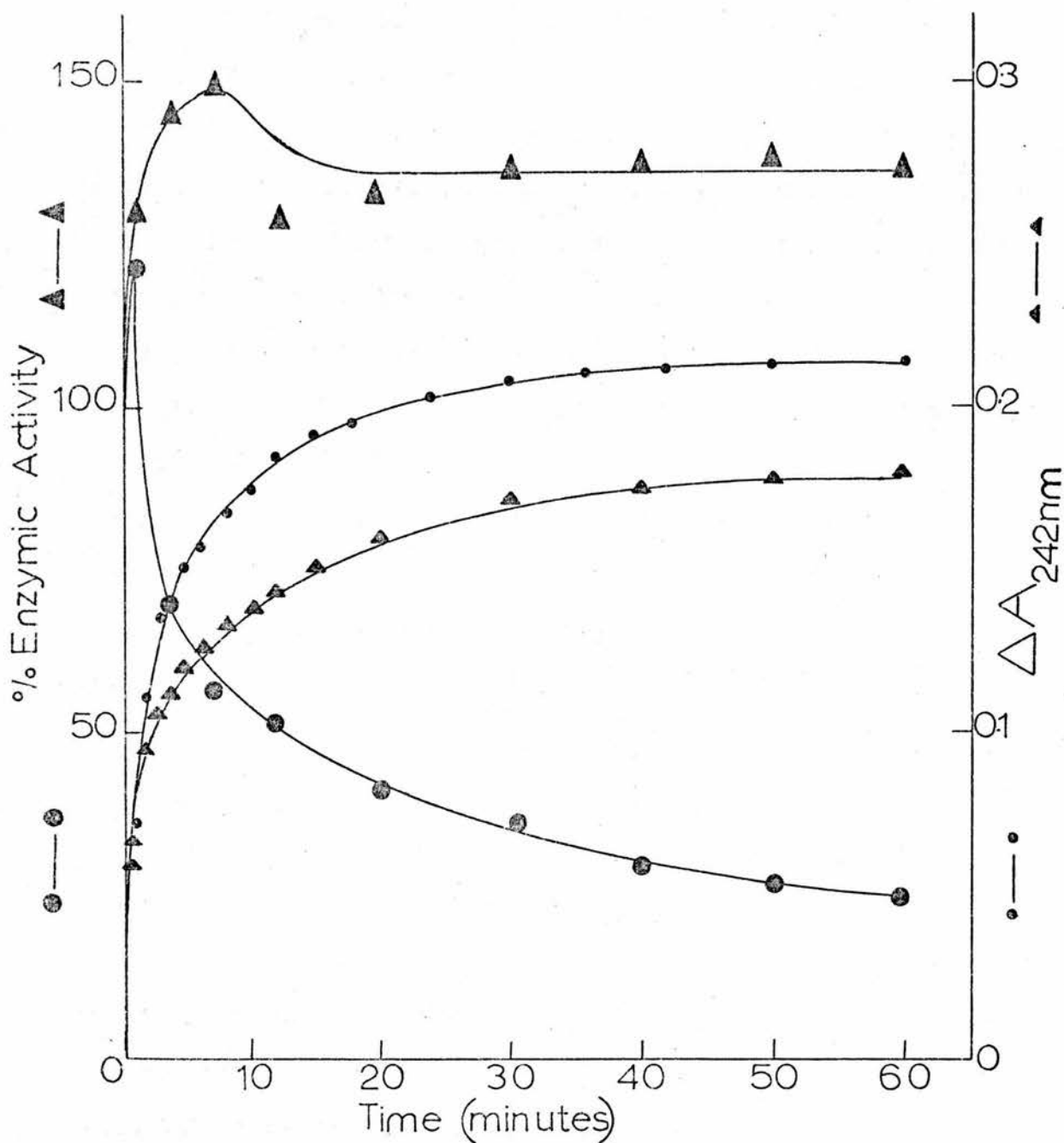


Fig.8-7. Relation of the extent of inactivation of LADH by DEP to the increase in absorbance at 242nm. Enzyme, 6.9 μ M; 0.1M phosphate buffer, pH 6.1; 23.5°C; DEP, 4.65mM; ●, without added ligand; ▲, ▲, + DPNH (13 μ M) and isobutyramide (10mM).

From Table 8-1 it can be seen that, although coenzyme protected against inactivation, nevertheless, the same number of histidine residues were carbethoxylated as in unprotected enzyme. Formation of the ternary complex, ER.IB also resulted in no protection of histidine. Four histidine residues per subunit were carbethoxylated giving rise to an activation of the enzyme, which was stabilized by the presence of coenzyme. Denaturation of the enzyme in 8 M urea or 0.5% sodium dodecyl sulphate failed to make all the histidine residues in the enzyme accessible to DEP. However, all the histidines of CM-LADH were carbethoxylated. This resulted in precipitation of the protein. Native enzyme began to precipitate slowly when incubated with DEP for five hours, but the ternary complex ER.IB showed no signs of precipitation after 20 hours, although the activity enhancement was diminished.

Fig. 7 shows that the rate of carbethoxylation of histidine in LADH, measured by following the increase in absorbance at 242nm, is very similar for native enzyme and the ternary complex ER.IB.

As shown in Table 8-2 the concentrations of coenzyme binding sites on the enzyme determined by fluorimetric titration with DPNH in the presence of excess isobutyramide, was unchanged on reaction of the enzyme with DEP.

Table 8-3 shows that removal of the N-carbethoxyl groups with hydroxylamine did not restore the enzyme to its original activity. The inactivation process appears to be an inevitable, irreversible consequence of the initial activation process due to carbethoxylation of

TABLE 8-1

The relation between the number of N-carbethoxyl-histidine residues and the activity of liver alcohol dehydrogenase.

LADH was incubated in 0.1M phosphate, pH 6.0, with a 21-fold molar excess of DEP. Enzyme, 4.00 - 5.00 μ M.

Incubation conditions	Time (hours)	% Activity	The number of N-carbethoxyl histidine residues per subunit
23.5°C	1.0	56	4.0
"	1.5	56	4.0
"	5.0	56	-
ER.1B, 23.5°C	1.0	224	3.9
	20.0	180	4.5
8M Urea, 0°C	1.5		4.7
"	4.0		4.9
0.5% SDS* 0°C	1.5		4.9
	4.0		5.7
ER, 23.5°C	1.0	158	4.3
	4.0	144	4.3
CM-LADH, 0°C	1.0		5.8
	4.0		7.3

* Sodium dodecyl sulphate

TABLE 8-2

The effect of carbethoxylation of liver alcohol dehydrogenase on the coenzyme binding site concentration.

The coenzyme binding site concentration of a solution of the enzyme in 0.1M phosphate, pH 6.0, 23.5°C was measured by diluting aliquots in 0.1M phosphate, pH 6.0, plus 50mM isobutyramide and titrating fluorimetrically with DPNH. A 20-fold molar excess of DEP was added to the incubation solution. After 60 minutes the enzyme activity and binding site concentration were determined. The number of histidine residues carbethoxylated per subunit was determined.

Time	Number of N-carbethoxyl groups per subunit	Coenzyme binding site concentration	% Activity
0	0	18.9 μ N	100
60	4.2	18.9 μ N	53

TABLE 8-3

The effect of the removal of the N-carbethoxyl groups of histidine on the activity of N-carbethoxyl-liver alcohol dehydrogenase.

All incubation solutions contained $13.9\mu\text{M}$ enzyme (E) in 0.1M phosphate, pH 6.0. With the exception of solution (1) they were incubated for one hour with a 20-fold molar excess of DEP. The enzyme activity of the solutions was measured and with the exception of solution (2) made 0.1M in hydroxylamine, and adjusted to pH 7.0. The solutions were then passed through a column of sephadex-G25 equilibrated with phosphate buffer, pH 7.0, ionic strength 0.1. The enzyme activity of the eluate was measured and the enzyme concentration determined from the absorbance at 280 nm. The extinction coefficient was assumed to be the same as for native enzyme.

Additions		Incubation solution	% Acti- vity after incubation	% Activity after elu- tion from the column
DEP	Hydroxyla- mine			
-	+	1. E	100	93
+	-	2. ER.1B	168	76
+	+	3. E	50	48
+	+	4. E	53	35
+	+	5. ER.1B	160	76

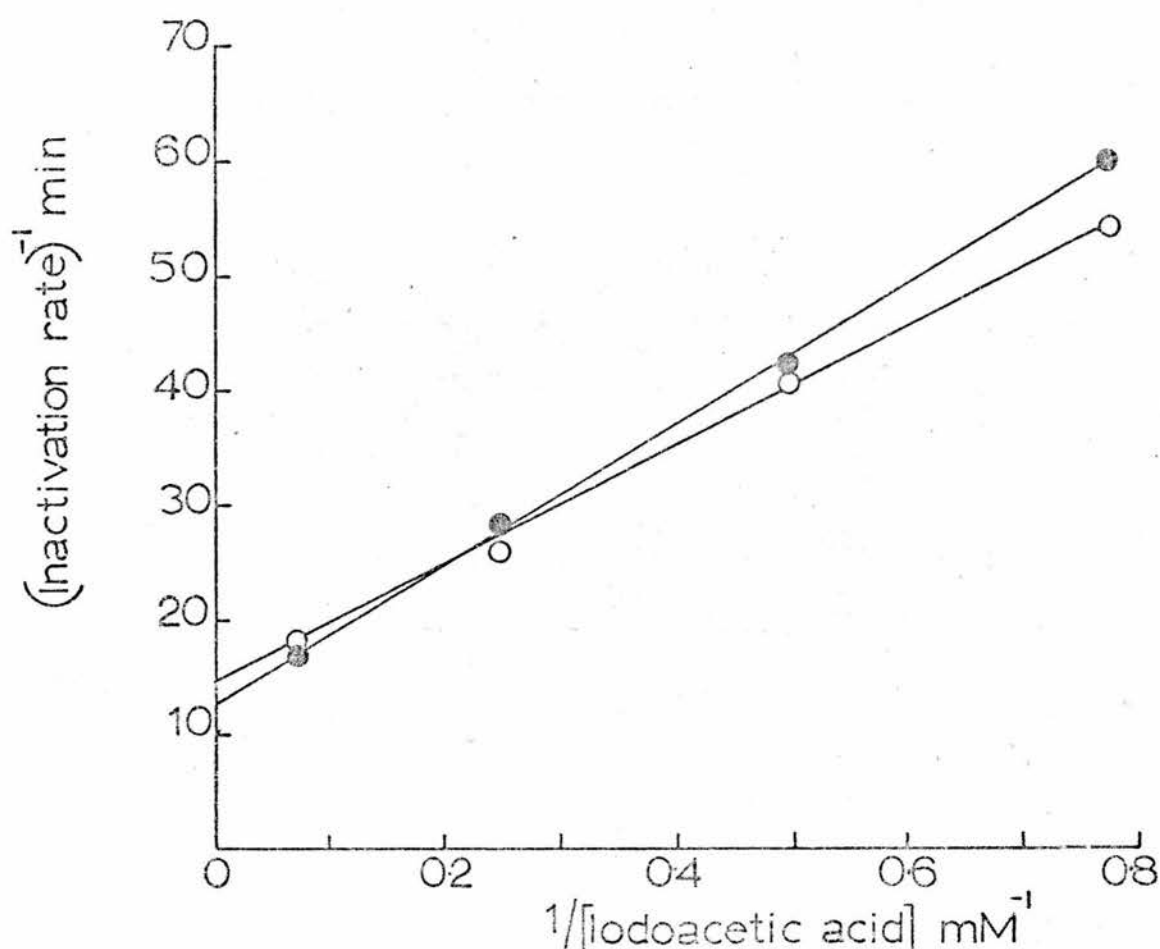


Fig.3-8. Double reciprocal plot showing the inactivation of N-carbethoxyl-LADH by iodoacetate. Phosphate buffer, pH 7.4, 23.5°C and ionic strength 0.1 (maintained by varying the phosphate concentration) o, native enzyme, 1.3 μM ; •, N-carbethoxyl-LADH, approximately 2 μM . (N-carbethoxyl-LADH was prepared by incubating for 60 minutes 45 μM LADH in 0.1M phosphate, pH 6.0, 23.5°C with a 20-fold molar excess of DEP. The modified enzyme was separated from excess reagent by passing through a column of G.25 sephadex (medium). Residual activity after eluting from the column was 45%.

four histidine residues per subunit. Supporting this it was found that removal of the N-carbethoxyl groups from activated DPNH protected enzyme, followed by removal of DPNH did not prevent the inactivation process.

The inactivation of N-carbethoxyl-LADH by iodoacetate follows Michaelis-Menten-type kinetics (fig. 8-8). The maximum rate of inactivation of the modified enzyme was $7.9 \times 10^{-2} \text{ min.}^{-1}$ compared to $6.5 \times 10^{-2} \text{ min.}^{-1}$ for the native enzyme. The reversible binding of iodoacetate is not significantly altered.

Discussion

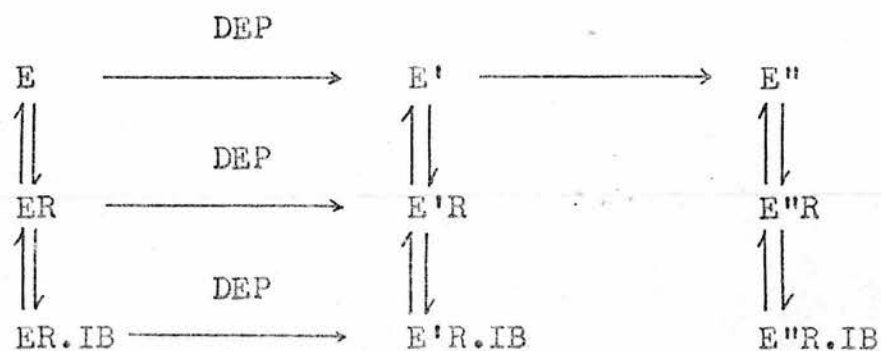
Four histidine residues per subunit of LADH are found to be readily accessible to N-carbethoxylation whereas three are 'buried'. Since none of the 'accessible' histidines are protected by DPNH or DPNH plus isobutyramide, they must be at sites on the enzyme surface other than the active centre. Thus they cannot be involved in binding substrate or coenzyme. For the same reason the suggestion that histidine plays an essential catalytic role in the alcohol dehydrogenase reaction (Ringlold, 1966) also has to be ruled out. If it did, such a residue would be expected to be in an accessible site at the active centre and be strongly protected by formation of ER.IB. Moreover, modification of such an essential histidine would lead to complete inactivation, whereas carbethoxylation of the accessible histidines of LADH leads to only partial inactivation.

Cysteine-46 occurs in a highly polar region of the amino acid sequence (Jörnvall and Harris, 1970) and is

presumably exposed at the surface of the enzyme. A histidine residue interacting with this thiol would also be accessible to a small neutral molecule such as DEP. However, since the rate of inactivation of N-carbethoxyl enzyme by iodoacetate is very similar to that of native enzyme, such an interaction is unlikely for carbethoxylation of a histidine in the above role would abolish or significantly alter the interaction.

Regarding the three 'buried' histidines it is possible that these are ligands to the active centre zinc, a role suggested previously for histidine residues (Theorell and McKinley-McKee, 1961c; Plane and Long, 1963). Denaturation of the enzyme failed to reveal all the histidines to DEP while all seven histidines per subunit were carbethoxylated in CM-LADH. Since carboxylmethyl-enzyme has labilized 'structural' zinc atoms (Drum et al., 1967), this may suggest that the 'buried' histidines are more likely to be ligands to these zinc atoms. However, once all seven histidines per subunit were carbethoxylated, CM-LADH precipitated. It is probable that the enzyme unfolded completely during carbethoxylation and both the 'active centre' and the 'structural' zinc atoms were lost, exposing all the zinc ligands. The 'structural' zinc ligands may be thiols rather than histidines since sulphur has also been thought to attach zinc to the enzyme (Ulmer and Vallee, 1965; Wallenfels and Sund, 1957).

Since inactivation of LADH by carbethoxylation is irreversible this suggests it may trigger off a series of conformation changes occurring as follows:



Here carbethoxylation of native enzyme (E) rapidly converts the enzyme to a new conformer (E'), which has enhanced activity, but which then more slowly changes to a conformer (E''), which has impaired activity. The enhanced activity of E' is perhaps due to greater lability of E'R, while the origin of the impaired activity of E'' is unknown. The binding of DPNH to LADH is considered to produce a profound conformational change since it has been shown that, whereas the enzyme itself crystallizes in orthorhombic symmetry the ER and ER.IB complexes crystallize in monoclinic or triclinic symmetry (Zappezauer et al., 1967). A considerable conformational change was also indicated by ORD spectra of the native enzyme and the ER.IB complex (Rosenberg et al., 1965). Thus DPNH and DPNH plus isobutyramide appear to prevent the postulated irreversible conformational change, (E' \longrightarrow E'') by holding the enzyme in a stable conformation. The partial protection afforded by AMP, decanoate and 4.biphenylcarboxylic acid indicates that they may form complexes with the enzyme that also maintain conformations resistant to the conversion of E' to E''. Bromide afforded no protection against inactivation and may produce a looser structure. This is con-

consistent with the increased rate of dissociation of DPNH from the enzyme in the presence of bromide (Theorell, Bonnichsen and Nygaard, 1955).

It is concluded that the 'accessible' histidines do not play a role in binding substrate or coenzyme, in catalysis or in activation of cysteine-46. However, the tertiary structure of the native enzyme is sensitive to modification of these histidines

Chapter 9.

General Discussion.

Concave-down Lineweaver-Burk plots

In this thesis one of the chief tools used in the investigation of the functions of various structural features of LADH, principally the 'active centre' zinc atom, lysine residues and histidine residues, was steady state kinetics in the presence of inhibitors. In the case of the thiols in particular very pronounced concave-down Lineweaver-Burk plots (L-B plots) were obtained. A summary of causes of concave-down L-B plots is presented and the relevance of each cause to the curves produced by the thiols is discussed.

- 1) Dalziel (1958b) derived by the steady state method the initial rate equation for the alternative pathway mechanism for a two substrate enzyme which included reversible transformation of the ternary complex to products and in which no restrictions were imposed. In the absence of products and with one substrate held at constant concentration, the double reciprocal form of this equation is,

$$1/v = \frac{a + b(1/s) + c(1/s)^2}{d + e(1/s)}, \text{ where}$$

v is the initial velocity, s is the concentration of the varied substrate, a , b , c , d and e are functions of rate constants and the constant concentration of substrate. This equation is a 2/1 function which are concave-up or down near the ordinate and have asymptotes as $(1/s)$ approaches infinity. Thus concave-up or down L-B plots are inherent in this mechanism. Cleland (1970) has

stated, however, that it has not been established experimentally that any observed cases of concave-up or down L-B plots are due to an enzyme having this kinetic mechanism. Silverstein and Boyer (1964) concluded that a small part of the LADH catalysed reaction takes place by the alternative pathway, with ethanol binding first. Thus it is conceivable that thiols may inactivate one active centre of the dimeric LADH molecule but alter the properties of the other active centre by an effect transmitted through the protein so that the alternative pathway becomes more important hence giving rise to concave-down L-B plots.

- 2) Inhibitors which have values for K_I of the same order as the enzyme concentration have been shown to lead to concave-down L-B plots (Morrison, 1969). This applies to competitive, non-competitive and un-competitive inhibition in a compulsory order bireactant mechanism. This type of inhibition also leads to plots of total inhibitor concentration against the reciprocal of the initial velocity which are concave-upwards. ME and ethanethiol were used in concentrations differing by a factor of 100 to produce the same magnitude of inhibition, which would tend to rule out the possibility that an impurity which could bind extremely tightly to LADH was present. Moreover, replotting the reciprocals of initial velocities at a given DPN^+ concentration against thiol concentration produces

a straight line rather than one concave-up.

- 3) Concave-down L-B plots are always found when two enzymes are present catalyzing the same reaction, but with different apparent michaelis constants. However, the LADH preparation used consisted very largely of the isoenzyme EE and straight L-B plots were always obtained in the absence of inhibitors.
- 4) Cleland and Wratten (1964) showed that LADH is inhibited by methanol and obtained concave-down L-B plots when ethanol or acetaldehyde was varied but straight L-B plots when DPN^+ or DPNH was varied. They showed that a scheme which included $\text{E} \cdot \text{DPN}^+$ inhibitor complexes which are not dead end but can combine with ethanol and be converted to $\text{E} \cdot \text{DPNH}$ -inhibitor.aldehyde complexes was one possible explanation of these observations. Since straight L-B plots were obtained when ethanol was varied in the presence of ethanethiol and the thiols appear to be strictly competitive with ethanol the existance of $\text{E} \cdot \text{DPN}^+ \cdot \text{ethanol} \cdot \text{thiol}$ complexes can be ruled out.
- 5) Negative co-operativity in the binding of substrates to enzymes was predicted from the sequential model of subunit interactions by Koshland and his co-workers who also produced evidence that ligand induced conformational changes were responsible for this phenomenon (Koshland, Nemethy and Filmer, 1966; Kirtley and Koshland, 1967). This phenomenon has been proposed as an explanation of certain

properties of a number of enzymes including, phosphoenolpyruvate carboxylase (Corwin and Fanning, 1968), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland, 1968), CTP-synthetase (Levitzki and Koshland, 1969) and glutamate dehydrogenase (Dalziel and Engel; 1968). One consequence of negative co-operativity in the binding of a substrate to an enzyme emphasised in the above work is the occurrence of concave-down L-B plots when the concentration of that substrate is varied. An explanation of the concave-down L-B plots produced when LADH is inhibited by thiols could involve thiol induced conformational changes in the protein in the manner discussed by the above workers. Suppose a thiol binds at the active centre of one subunit of LADH and induces a conformational change in that subunit which through subunit interaction alters the conformation of the second subunit thus changing the properties of the active centre in the second subunit. Now the enzyme can be considered as a mixture of dimeric molecules with, (a) no inhibitor bound and both active centres functioning normally, (b) one inhibitor molecule bound blocking one active centre and altering the properties of the second active centre via subunit interaction, (c) two inhibitor molecules bound and both active centres blocked. In the presence of the inhibitor the enzyme would behave as a mixture of two enzymes catalyzing

the same reaction but with different apparent michaelis constants for substrate. This as mentioned in category (3) above would give rise to concave-down L-B plots. As it is well established that the formation of EO.pyrazole and ER.isobutyramide is accompanied by profound conformational changes in the protein it is quite possible that EO.thiol formation also produces conformational changes. However, there is no indication that concave-down L-B plots are produced when LADH is inhibited by pyrazole or isobutyramide (Theorell and McKinley-McKee, 1961 c; Theorell et al., 1969).

A considerable amount of evidence has suggested that the active centres of LADH are equal and independent. Theorell et al., (1970) have even presented evidence that the equilibrium and rate constants for the binding of DPNH to ES are exactly intermediate between those for EE and SS. On the other hand some evidence has been accumulated that under certain conditions the subunits may not be independent. The zinc free apoenzyme although inactive binds DPNH at two non-identical binding sites (Hoagstrom et al., 1969). Only one DPNH molecule per LADH molecule reduced the line broadening of ^{35}Cl in NMR experiments, but this increased to two in the presence of isobutyramide (Lindman et al., 1970). This particular result is doubtful since Ward and Happe (1971) in similar experiments found that two DPNH molecules reduced the line broadening in the absence of isobutyramide. Rapid reaction kinetics by Bernhard et al., (1970) using aromatic substrates showed that when substrates

were in excess there was a rapid 'burst' of product formation equivalent to half the number of active centres, but when enzyme was in excess of one of the substrates there was a 'burst' equivalent to half of the limiting substrates. It was suggested that subunit interactions were occurring. Finally, Theorell and Tatemoto (1971) from studies on the affect of coenzyme and other ligands on the protein fluorescence of the enzyme suggested that excitation energy can be transferred between the subunits. Thus the question of whether or not conformational changes can be transmitted between the subunits of LADH has not been finally settled. The possibility that the binding of thiol to EO produces a conformational change which is transmitted between the subunits cannot therefore be dismissed. The concave-down L-B plots observed in the presence of thiol may be produced by pH dependent conformational changes induced by thiol binding to EO which have a major effect on DPNH binding to the other subunit near neutral pH but not at alkaline pH and vice versa for DPN^+ binding.

The 'active centre' zinc atom and the substrate binding site

Thiols were found to be strictly competitive with ethanol and since the strength of their binding to EO increased with increasing hydrophobicity of the thiol it is probable that the aliphatic part of the thiols and ethanol compete for the same binding site. Thus even if it was certain that the thiol group was bound by the zinc atom competition of thiol with ethanol would not prove that

the zinc atom is also binding the hydroxyl group of ethanol. Moreover, if the binding sites for ethanol and thiol in the active centre of the EO complex are distinct, conformational changes produced by these compounds on binding could exclude the other thus giving rise to competition. The hydroxyl group of ethanol may interact with both the active centre zinc and the nicotinamide ring of DPN^+ . Some evidence indicates that this is reasonable. Mildvan and Weiner (1969) and Mildvan (1969) estimated that the substrate binding site is $\leq 5\text{\AA}$ from the nicotinamide ring of DPN^+ by using a paramagnetic analog of DPN^+ (ADPR). Further, by comparison of the enhancement of the rate of proton relaxation with zinc free apoenzyme ADPR complex with native enzyme ADPR complex, they suggest that the active centre zinc immobilizes a water molecule near the unpaired electron of ADPR and that ethanol and acetaldehyde are also bound near this electron.

A possibility which must be considered in the light of some recent evidence is that zinc is not in the active centre. X-ray crystallographic analysis of LADH has revealed only one zinc atom per subunit which is about 25\AA from the coenzyme binding site (Brandén et al., 1969). Young and Wang (1971) have interpreted the results of their experiments with LADH in which the 'active centre' zinc had been replaced by cobalt as showing that orthophenanthroline and the substrate competitive inhibitors azide and pyrazole do not bind to the 'active centre' zinc. The experiments with thiols indicate that ligands can form very tight EOI complexes, in which

hydrophobic interaction with the enzyme plays an important part, without necessarily binding to the active centre zinc. It is known, for example, that zinc in the active centre of carbonic anhydrase does not bind the substrate, carbon dioxide (Davis, 1961). The function of the conformational changes observed on the binding of inhibitors to EO and ER may be to bring the 'active centre' zinc atom from outside the active centre into a position where it can act as a general acid catalyst towards bound substrate.

A consideration arising from the chemical modification of LADH with DEP is the nature of the ligands binding the active centre zinc to the enzyme. It is possible to prepare LADH which has lost the 'active centre' zinc atoms but retains the 'structural' zinc atoms by dialysing against diethyldithiocarbamate (Drum et al., 1969). However, comparison of the number of histidine residues modified by DEP in native enzyme and 'active centre' zinc free enzyme would not be a valid approach to identifying the 'active centre' zinc ligands. Removal of this zinc may well be accompanied by structural changes which could reveal hitherto inaccessible histidine residues and the apoenzyme could undergo similar changes during carbethoxylation. The three 'buried' histidine residues observed in chapter 8 are, however, obvious candidates for ligands from the protein to the 'active centre' zinc. Histidine has been found to be a predominant protein to zinc ligand in zinc containing proteins. Three histidine ligands have been implicated in the binding of zinc to alkaline phosphatase by photooxidation (Tait and Vallee,

1969), a glutamate and two histidine residues bind zinc to carboxypeptidase A as shown by the X-ray crystallographic structure and amino acid sequence (Lipscomb et al., 1969; Bradshaw et al., 1969); all three zinc ligands are histidine in human carbonic anhydrase C as shown by X-ray crystallography (Liljas et al., 1972).

It is apparent that the X-ray crystallographic structure of LADH, binary complexes of LADH with coenzyme and ternary complexes EO pyrazole and ER isobutyramide, in which all four zinc atoms are identified, will have to be awaited before the problem of the role of zinc in binding substrate and the nature of the protein to zinc ligands are resolved.

Chemical Modification of LADH

The experiments with the thiols produced little evidence as to the chemical nature of the binding sites of the thiols or substrates in the active centre. Experiments with LADH modified by removal of the active centre zinc or by replacement of the active centre zinc by other metals as in the work of Drum (1970) and Young and Wang (1971) should provide worthwhile results. However, the chemical modification experiments designed to investigate the role of lysine and histidine residues in LADH has provided useful information.

Based on the results obtained from chemical

modification of the lysines of LADH presented in this thesis is the suggestion that one lysine at the active centre of the enzyme helps to bind coenzyme by attracting the phosphate of the AMP moiety. One must, however, exercise caution when the bulk of the evidence for such a postulation is drawn from ligand protection studies. This can be illustrated by reference to previous work on the thiols of various dehydrogenases. The 'essential' thiols of liver ADH, yeast ADH and lactate dehydrogenase are completely protected from blocking with thiol reagents by coenzyme and this has sometimes been taken to mean that these thiols are involved in binding coenzyme, (e.g. Rabin and Whitehead, 1962; Whitehead and Rabin, 1964). However, it has subsequently been shown that modification of the 'essential' thiols of LADH by iodoacetate (Li and Vallee, 1965; Reynolds and McKinley-McKee, 1970); of yeast ADH by iodoacetamide (Dickinson, 1972) and of lactate dehydrogenase by maleimide (Holbrook, 1966; Holbrook and Stinson, 1970) results in no loss of coenzyme binding capacity. Thus it appears in these cases that the 'essential' thiols are not at the coenzyme binding site. This has been verified for dog fish lactate dehydrogenase (M_4) by X-ray crystallography which shows that the 'essential' thiol is 13.5 Å from the nearest part of the bound coenzyme molecule (Adams et al., 1970 a, b).

The possibility always exists that a ligand protects an amino acid residue from attack by a reagent by producing a conformational change in the protein which renders the amino acid inaccessible to that particular

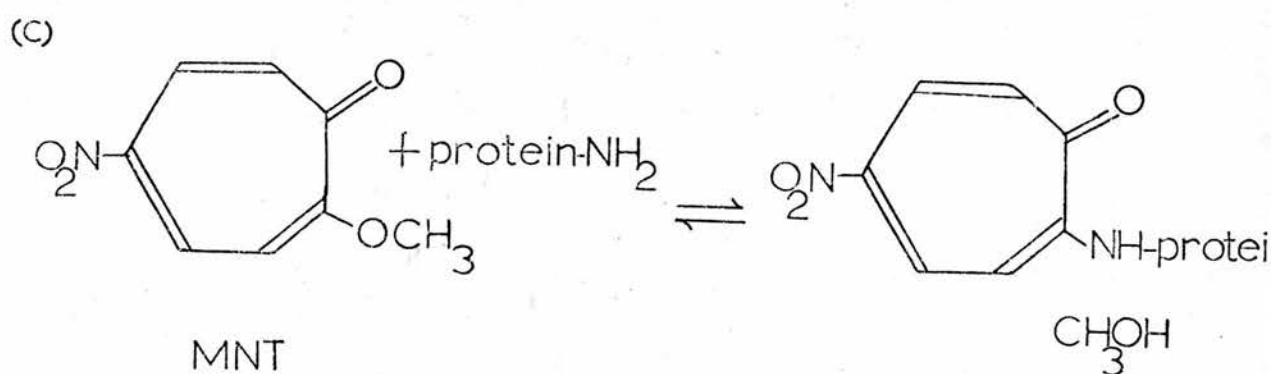
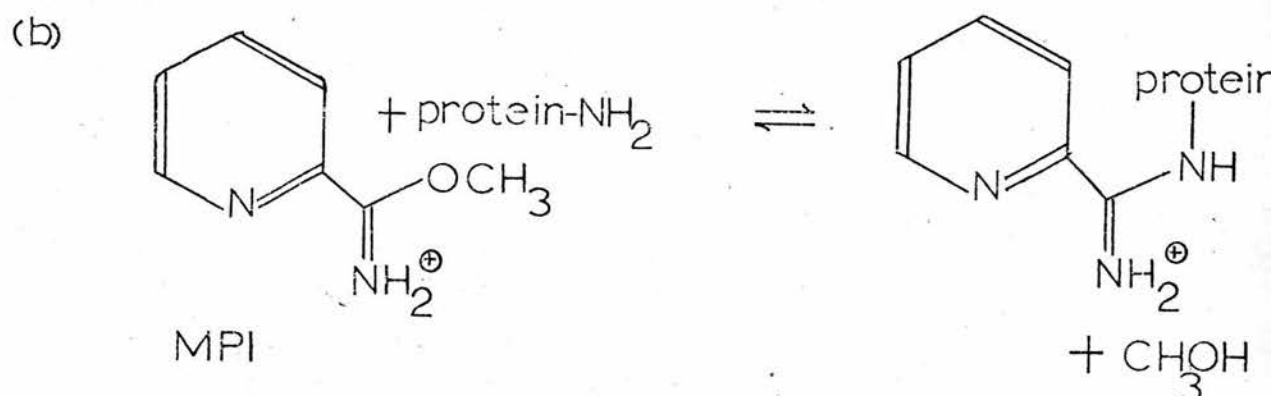
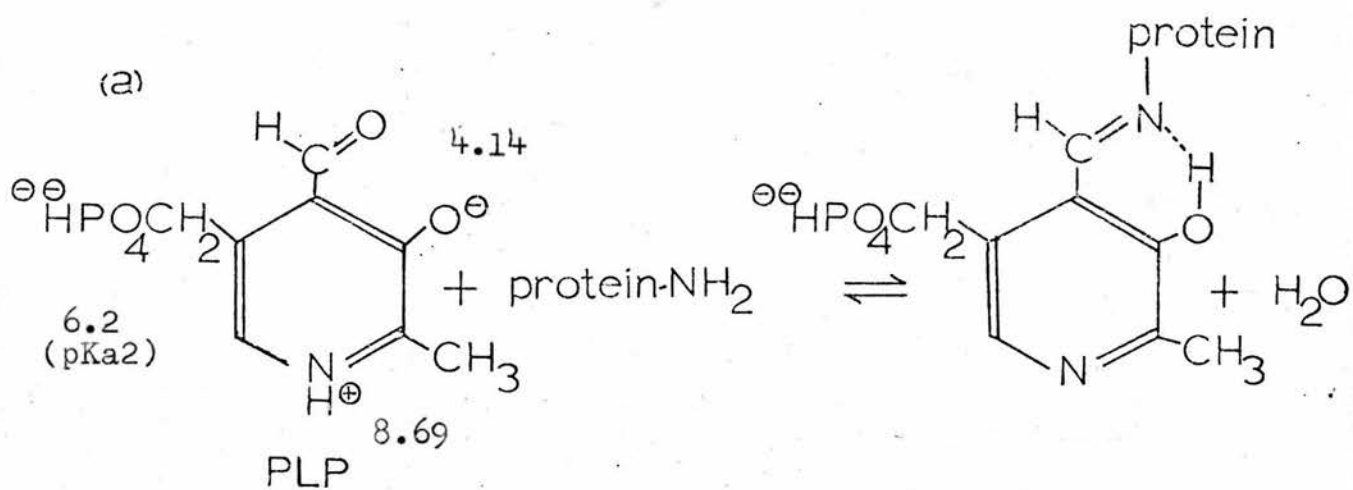


Fig.9-1. The reaction of (a) PLP, (b) MPI and (c) MNT, with amino groups in proteins. The predominant ionic species at pH 8.0 are shown with the exception of protein -NH₂ which is the reactive species in these reactions. Williams and Niellands (1954) have estimated the dissociation constants of PLP and pKa values are inserted adjacent to the appropriate group above.

reagent, rather than by binding directly over the amino acid residue. This problem can be tackled by studying how different ligands protect against modification, using different reagents to modify the amino acid and by investigating the properties of the modified enzyme. In the study of the lysines in LADH the reagents illustrated in fig. 9-1 were used, which enabled the positive charge of the lysine residues to be retained (MPl), or replaced by a negative charge (PLP) or a neutral residue (MNT). In all three cases a considerable increase in the bulk of the lysine residues was achieved. To establish that a lysine in the active centre is forming an ion pair with the phosphate of the AMP moiety of coenzyme it is necessary to show that:

- (a) AMP and coenzyme protect against inactivation by PLP and activation by MPl and MNT.
- (b) AMP and coenzyme do not bind to PLP-LADH to account for inactivation and their binding to picoliniminidated-LADH and nitroponyl-LADH is weakened to account for enhancement of activity.

Condition (a) is fulfilled for the three reagents. Plapp (1970) has shown that the binding of DPN^+ and DPNH to LADH is considerably weakened by picolinimidation and this study shows that the binding of AMP is also weakened. The high absorbance and fluorescence of PLP-LADH made spectrophotometric or fluorimetric determinations of coenzyme binding impracticable whereas the nitroponylation of LADH resulted in an inactivation process concomitant with activity enhancement. However, the fact that the

above conditions were fulfilled where tested and the finding that in general ligands which are thought to bind at the AMP binding site protected strongly against inactivation or activation, whereas other ligands did not protect or did so in a complex manner, strongly supports the conclusion that the protected lysine is situated in the active centre at the AMP binding site. The results also establish that this positive centre is not essential for coenzyme binding and the coenzyme can accommodate considerable steric interference and still form a catalytically active binary complex.

The finding that there are, perhaps, two PLP binding sites, the 'active centre' lysine and another site which is, perhaps not a lysine, at which PLP binding is mutually exclusive is not a unique situation. It has been shown the modification by iodoacetate of histidine-119 and histidine-12 in ribonuclease is mutually exclusive (Crestfield, Stein and Moore, 1963).

The interpretation of the limited incorporation of PLP into LADH in terms of electrostatic interaction of the phosphate group of PLP with charged groups on the enzyme may be the best approach. Arginine decarboxylase and glutamine decarboxylase both employ PLP as a coenzyme and it has been shown that the lysine which binds PLP to these enzymes, occurs in the amino acid sequence adjacent to a lysine and arginine residue (Boeker, Fischer and Snell, 1971). Thus in these enzymes, in which the binding site for PLP is designed specifically for that task, the formation of an ion pair between the phosphate of PLP

and a positive charge in the enzyme is probably a significant factor in PLP binding.

Further studies involving chemical modification of specific amino acid residues in LADH other than cysteine, lysine or histidine residues will be of value. However, the thiol content of LADH, particularly the presence of the reactive cysteine-46, and the instability of the enzyme below pH 6.0 and above pH 10 makes this a difficult task. A consideration of the reagents most selective for some amino acid residues emphasises the difficulties.

Carboxylic acids: Activation by water soluble carbodiimides (Hoare and Koshland, 1967) or isoxazolium salts (Bodlaender et al., 1969) followed by reaction of the activated carboxyl group with a nucleophile is specific for carboxyl groups only below pH 5.

Tyrosine: Nitration at the three position of tyrosine was shown to be achieved selectively with tetranitromethane at pH 8.0 but reaction with thiol groups in protein can also occur (Sokolovsky et al., 1966, 1969).

Tryptophan: Koshland et al., (1964) have developed a reagent, 2-hydroxy-5-nitrobenzyl bromide, which is highly selective for tryptophan residues at neutral or acidic pH in the absence of thiols.

Arginine: Phenylglyoxal has been introduced as a reagent which is specific for arginine in proteins at about pH 8.5 (Takahashi, 1963). This reagent has been used to show that one arginine per subunit of lactate dehydrogenase is involved in substrate binding (Berghäuser and Falderbaum,

1971). Modified arginine is not converted to arginine by acid hydrolysis so this reagent would seem promising for modification studies of LADH.

Histidine: Apart from DEP the most selective reagent devised for histidine is diazonium-1H-tetrazole (Horinishi et al., 1964) which has the disadvantage of reacting simultaneously with tyrosine residues, although in many cases reaction with histidine is complete before reaction with tyrosine begins.

The development of new reagents specific for the above amino acids can be seen to be necessary. Ideally it is important to use several reagents specific for a given amino acid so that groups of different electrostatic charge, stereochemistry and mechanisms of reaction can be inserted into the protein. Also it is by variation of these factors that, due to the protein environments of the amino acid residues being investigated, different reagents can be found which are specific for a restricted number of a given type of amino acid residues in a protein.

Conformational changes in LADH

When a ligand binds to a protein or an amino acid residue is chemically modified there will almost certainly be some degree of conformation change induced in the protein, even if only at a subtle and localized level. Thus the effect of ligand binding on the rate of extent of chemical modification studied in this work can in many cases be interpreted in terms of conformational changes produced in LADH by ligand binding. Some evidence was obtained that each of the following ligands produce a

conformational change in the enzyme.

DPNH: DPNH considerably reduced the extent of incorporation of nitrotroponyl residues into LADH. There is of course good evidence from previous work that DPNH produces a profound conformational change (Rosenberg et al., 1965, Zeppezauer et al., 1967).

AMP: The incorporation of PLP groups into LADH is increased by AMP. The binding of AMP to lactate dehydrogenase has been shown to produce a conformational change which generates a binding site for nicotinamide mononucleotide (McPherson, 1970).

Halide anions: Both chloride and bromide increased the incorporation of PLP into LADH. These anions might have been expected to reduce the number of PLP groups incorporated by competitive binding at the positive centres on the enzyme with which the phosphate groups of bound PLP can interact. Alternatively the high ionic strength (>1.0) prevailing in these experiments may have increased the binding of PLP at lysine residues in the vicinity of carboxyl groups by reducing electrostatic repulsion. However, this effect would also decrease the ion pair formation between the phosphate of PLP and positive sites on the enzyme thus tending to reduce incorporation of PLP. Thus, on balance the high ionic strength does not explain the increase in PLP incorporation and a conformational change may occur.

Imidazole: The evidence for an imidazole induced conformational change in LADH is stronger than for most other ligands. The rate of activation by MP1 was increased

and the rate and extent of incorporation of nitrotroponyl residues into LADH was increased. Also in the presence of imidazole the nature of the binding of PLP to the active centre lysine was altered although imidazole does not interfere sterically with this binding site. Previous work has shown that imidazole accelerates the rate of inactivation of LADH by iodoacetate (Evans and Rabin, 1968; Reynolds et al., 1970) and that the binding of DPNH is weakened by the presence of imidazole (Theorell and McKinley-McKee, 1961 b, c). Both these results have been interpreted in terms of conformational changes.

Orthophenanthroline: The nature of the binding of PLP to the active centre lysine is altered and the rate of activation of the enzyme by MPL is reduced although orthophenanthroline bound to the active centre zinc does not sterically hinder ligand binding at the 'active centre' lysine. In the case of MPL it is possible that orthophenanthroline displaces OH^- ions bound by the 'active centre' zinc thus increasing repulsion between positively charged MPL molecules and the zinc and thereby decreasing the rate of reaction of MPL with the active centre lysine.

The active centre of LADH

The difference between the binding of fatty and aromatic acids suggest an active site in which there are two distinct hydrophobic regions. Decanoate is competitive with 2,2-bipyridyl which binds to the 'active centre' zinc whereas BPCA is not (Sigman, 1967). The finding that BPCA and orthophenanthroline bind non-

competitively (fig. 7-10) supports the suggestion that decanoate binds to the 'active centre' zinc whereas BPCA does not. However, both decanoate and BPCA form EOI complexes. Thus it is probable that DPN^+ produces a change in the shape of the hydrophobic substrate site so that BPCA can be accommodated. An alternative explanation is that neither of these anions binds to zinc in the EOI complex. BPCA was calculated to have a K_{EOI} $108 \mu\text{M}$ (Chapter 7), whereas decanoate has a $K_{\text{EO,I}}$ of $2 \mu\text{M}$ (Theorell and McKinley-McKee, 1961 c). Thus although BPCA and decanoate have hydrophobic tails of comparable length the aliphatic tail of decanoate binds by far the more strongly. This suggests that in the ternary complex the substrate binding site is a narrow hydrophobic 'trench' into which the flat biphenyl tail of BPCA can only partially fit. The reverse situation occurs at the AMP binding site where BPCA is strongly competitive with coenzyme while decanoate does not seem to bind at this site. The biphenyl tail probably binds in a manner analogous to the adenosine portion of coenzyme with the carboxylate group attracted to the active centre lysine. Phenylacetic acid binds much more weakly at this site which can thus be assumed to be hydrophobic, and flat and exposed in comparison to the substrate binding site. It may contain tryptophan and/or phenylalanine residues which aromatic ring systems can 'stack' against in a manner analogous to the 'stacking' of the bases in the double helix of DNA.

The active centre lysine must lie adjacent to the hydrophobic adenine site. It would not be safe to assume

that the pK of this group is in the normal range for lysine ϵ ammonium groups in proteins since it has recently been demonstrated that a lysine in the active centre of acetoacetate decarboxylase has the abnormally low pK of 5.9. However, the pH dependence of the equilibrium value of % inactivation of LADH by PLP has a maximum at about pH 8.5. Since the unprotonated amino group is the reactive form of the lysine residue and the above maximum arises from an increase in the concentration of the unprotonated amino group conflicting with a decrease in the concentration of the protonated form of the aldehyde group of PLP as the pH is raised. The pK of the active centre amino group is not far above 8.5. This sort of value is much the same as the pK determined for a lysine, thought to be implicated in binding DPNH to glutamate dehydrogenase by the use of PLP and pyridoxal (Piskiewicz and Smith, 1971).

One can propose a sketch of the active centre in which there is a zinc atom bound to the enzyme by histidine residues with a hydrophobic 'trench' nearby, which is the substrate binding site. There is an aromatic hydrophobic site for binding the adenosine moiety of the coenzyme with a lysine immediately adjacent which is in a position to form an ion pair with the phosphate of the AMP moiety of bound coenzyme. Little is known about the nature of the other amino acid residues present in the active centre. Histidine appears to be absent. However, cysteine may be present since, although cysteine-46, which is reactive towards

iodoacetate, does not appear to be in the active centre, this does not rule out the presence of other thiol groups. It is very probable that at least one thiol group is situated there for LADH has been inactivated by covalent labelling with analogues of DPN^+ which bind to the enzyme competitively with coenzyme. An analogue of DPN^+ in which the nicotinamide moiety is replaced by 5-hydroxyl-4-methyl-thiazole is thought to form a disulphide bond with the protein (Van Eys et al., 1962). Analogues in which the nicotinamide moiety is replaced by a 3-(4-bromoacetylpyridinio) propyl group (an analogue with a 3-(3-bromoacetylpyridinio) propyl group inhibited the enzyme but did not inactivate) or the adenosine moiety replaced by 5-bromoacetyl-4-methylimidazole inactivated the enzyme and abolished DPNH binding (Woenckhaus et al., 1971, 1972). Thus it may be that there are thiols near both the nicotinamide and adenosine moiety binding sites. A thiol at the nicotinamide moiety binding site could be important in order to form a hydrogen bond with the carbonyl of the amide group of coenzyme which may explain why the 3-(3-bromoacetylpyridinio) propyl- DPN^+ analogue fails to inactivate LADH.

References

The following journal titles have been abbreviated:

ABB,	Arch. Biochem. Biophys.
ACS,	Acta. Chem. Scand.
BBA,	Biochem. Biophys. Acta.
BJ,	Biochem. J.
BBRC,	Biochem. Biophys. Res. Commun.
EJB,	Eur. J. Biochem.
JACS,	J. Am. Chem. Soc.
JBC,	J. Biol. Chem.
PNAS,	Proc. Nat. Acad. Sci. U.S.A.

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Appendix

Some of the work described in this thesis has been published in the European Journal of Biochemistry.

- (a) The results described in Chapter 4 have been published as a contribution to a paper (Reynolds, C.H., Morris, D.L., and McKinley-McKee, J.S. (1970) 14 14.)
- (b) A paper entitled "The Lysines in Liver Alcohol Dehydrogenase. Chemical modification with pyridoxal-5'-phosphate and methylpicolinimidate, composed by this author describes the major part of the results presented in Chapters 5 and 6. (McKinley-McKee, J.S., and Morris, D.L. (1972) EJB. 28 1).
- (c) A paper entitled "The Histidine in Liver Alcohol Dehydrogenase. Chemical modification with Diethylpyrocarbonate", composed by this author, describes the results presented in Chapter 8. (Morris, D.L., and McKinley-McKee, J.S. (1972) EJB. 29 515).

Reprints of the two papers mentioned in (b) and (c) above are bound in the back of the thesis.